IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.	:	10/796,113
Applicant	:	SPECTOR
Filed	:	March 10, 2004
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DECLARATION OF DR. MYRON SPECTOR

Director of the United States Patent
and Trademark Office
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

1. I am the same Myron Spector named as an inventor in the above-referenced U.S. patent application.
2. A copy of my curriculum vitae is attached hereto as Exhibit A. In addition to my current academic appointments including Professor of Orthopedic Surgery (Biomaterials) at Harvard Medical School, and my major visiting appointments as listed in my curriculum vitae, I have been conducting research on nerve regeneration tubes for over a decade, and have conducted research on BioGide® (Geistlich Biomaterials, Wolhusen, Switzerland) for about the last 7-8 years. I am an inventor in U.S. Patent No. 6,352,558 which includes utilization of BioGide® in a method for promoting regeneration of surface cartilage in a damaged joint, and I am also an inventor in numerous other patents and patent applications.
3. I have been informed that the claims of the above-referenced patent application has been rejected as being obvious over various combinations of Geistlich et al., U.S. Patent No. 5,837,278, Shimizu U.S. Patent No. 6,090,117, abstracts to Hentz et al. and Rosen et al., Stensaas U.S. Patent No. 4,778,467 and Humes U.S. Patent No. 5,429,938. I have reviewed these references and am familiar with their contents.
4. The present claims of this application are directed to a nerve regeneration tube and methods for reconnecting nerve ends, in which the tube has a resorbable side wall formed with collagen sheet material having a compact, smooth outer barrier surface, the sheet material further having a soft fibrous inner surface opposite the smooth barrier surface. The tube has an outer surface formed by the compact smooth outer barrier surface so as to inhibit cell adhesion thereon

and act as a barrier to prevent passage of cells therethrough. The tube further has an inner surface formed of the soft fibrous inner surface of the collagen sheet material, for promoting nerve growth. The ends of damaged nerves are positioned within opposite ends of the tube during use, for reconnection of the nerve ends. The nerve regeneration tube avoids formation of scar tissue which impairs nerve healing.

5. The surface configuration of tubes defined by the present claims provides such tubes with unexpected properties which could not have been predicted based upon the prior art. As indicated above, I have been researching nerve regeneration tubes for more than a decade. During earlier research that I participated in, comparisons were made between collagen nerve regeneration tubes and silicone nerve regeneration tubes, see, e.g., the papers attached hereto as Exhibit B, Chamberlain et al., Histological response to a fully degradable collagen device implanted in a gap in a rat sciatic nerve, *Tissue Engineering*, 3,4:353-362, 1997, and Exhibit C, Chamberlain et al., Connective tissue response to tubular implants for peripheral nerve regeneration: the role of myofibroblasts, *J. Comp. Neurol.* 417:415-430, 2000. The collagen tubes that were used in these earlier studies were obtained from Integra Life Sciences (Integra), Plainsboro, NJ, and are fabricated by freeze-drying Type I microfibrillar collagen from bovine tendon. The Integra collagen tubes are formed by collagen slurry injection over glass rods, and do not have a soft fibrous inner surface, a fact that I did not consider relevant at the time. In the earlier studies reported in Exhibits B and C, the Integra tubes were compared to silicone nerve regeneration tubes using a severed sciatic nerve rat model. Both studies show that the silicone tubes resulted in substantially greater build-up of fibrous scar tissue within the tubes, as compared to the Integra collagen tubes, with the Exhibit C study indicating that the silicone tubes resulted in formation of a fibrous capsule 10 times thicker than in the Integra collagen tubes. The problem with such fibrous build-up is that this fibrous tissue contains contractile fibroblasts (myofibroblasts) which cause the contracture of the fibrous layer. The contracting fibrous cuff interferes with the elongation of axons through the tube, and thus interferes with nerve regeneration. Although I did not recognize the significance at the time, the silicone tubes have a smoother inner surface than the Integra collagen tubes. Instead, at the time, we noted in the Exhibit C report that: "The differences in connective tissue response between collagen and silicone tubes could have been due to their known differences in chemical composition, permeability, or degradability." My subsequent research and analysis indicates the thickness of the fibrous scar which forms along the inner surface of the tube is related to the topography of the surface, with smoother surfaces favoring the formation of a thicker scar layer with a great number of contractile cells.

6. The Shimizu patent discloses 3-layer tubes which have smooth collagen or gelatin inner surfaces. Shimizu discloses from column 6, line 48 to column 7, line 50 thereof, formation of a 3-layer collagen tube. A central collagen layer 21 initially is formed on a Teflon rod. This central collagen layer 21 is compressed

into a high density, fine fibrous collagen layer, which necessarily and inherently imparts layer 21 with a smooth interior surface. After compression, the central layer 21 is removed from the Teflon rod, and the central layer 21 is repeatedly dipped into a hydrochloric acid solution containing collagen, to deposit collagen hydrochloric acid solution layers 22 and 23 on the inner and outer surfaces of the compressed collagen layer 21. The repeated dipping and drying procedure into collagen hydrochloric acid solution necessarily and inherently forms smooth amorphous inner and outer surface layers 22 and 23 on the compressed central layer 21 of the tube. The same result will necessarily and inherently be obtained if gelatin instead of collagen is utilized for the inner and outer surface layers. Under no conditions disclosed in Shimizu will a soft fibrous inner surface be formed.

7. Based on my studies and experience, the smooth inner surface that will be produced according to the methods of Shimizu will promote formation of a thick layer of fibrous scar tissue on the inner smooth surface of the tube, containing contractile fibroblasts (myofibroblasts) which cause contracture of the fibrous layer and interference with nerve regeneration.

8. The Geistlich et al. patent discloses a resorbable collagen membrane which is surgically inserted around the periphery of a wound cavity to facilitate, e.g., bone regeneration. In view of this reference, when combined with the other applied references, persons of ordinary skill in the art could not have predicted the unexpected results which have been achieved with the present invention, as outlined below.

9. Attached hereto as Exhibit D is a summary of a study that I was involved in, and which was presented at the 2007 Society for Biomaterials meeting. The Exhibit D study compares results achieved in five groups of animals (Groups I-V) in a rat spinal cord model for nerve regeneration. The study included testing of the collagen tubes (Groups III and IV) which we fabricated by freeze drying Type I microfibrillar collagen from bovine tendon from Integra, after slurry injection of the collagen over a glass rod mandrel. These tubes do not have a soft fibrous inner surface.

10. The Exhibit D study included testing of BioGide® collagen membrane (Group V) from Geistlich Biomaterials, Wolhusen, Switzerland. This BioGide® collagen membrane material corresponds exactly to the BioGide® collagen sheet material exemplified in the present application and usable in accordance with the present claims. The BioGide® membrane sheet material utilized in Group V of the Exhibit D study has a compact smooth outer barrier surface and a soft fibrous inner surface. In Group V of the Exhibit D study, the tube was formed by wrapping BioGide® membrane sheet material around stump ends of severed spinal nerves, so as to form a nerve regeneration tube as set forth in the present claims, with the soft fibrous surface oriented inwardly toward the severed nerve tissue to form the inner surface of the tube.

11. In the Exhibit D study, the Group V animals with tubes formed of Geistlich BioGide® membrane material having a smooth outer surface and a soft fibrous

inner surface, unpredictably had the highest number of axons in the center of the nerve defect, see, Figure 1 in Exhibit D.

12. In the Exhibit D study, the only difference between the Group V animals and the Group IV animals was the structure of the tubular material surrounding the severed nerve tissue. The "dorsal barrier" mentioned in the Exhibit D study refers to a collagen membrane draped over the implant site to assist in preventing overlying tissue (e.g., muscle) from collapsing into the nerve defect.

13. Taking into consideration the difference in the tube structure alone, between the Group V and Group IV animals, persons of ordinary skill in the art could not have predicted that the presently claimed invention, utilizing the collagen membrane material of Geistlich et al. U.S. Patent No. 5,837,278 (Group V), could result in the expectedly highest number of center nerve axons among the test animals, as compared to collagen tubes without a soft fibrous inner surface (the Group IV tubes).

14. With reference now to Exhibit E attached hereto, Fig. 1 thereof shows a cross-section through the BioGide® collagen membrane material with the compact smooth barrier side at the top, and the soft fibrous side at the bottom. As shown in Fig. 2 of Exhibit E, entubulation of a gap in a rat nerve (spinal cord) with BioGide® demonstrated the absence of a thick fibrous scar on the inner surface of the tube, and demonstrated the ingrowth of cells and tissue into the soft fibrous surface. Based on the prior art, persons of ordinary skill in the art could not have predicted the absence of a thick fibrous scar on the inner surface of a tube according to the present invention, in conjunction with ingrowth of cells and tissues into the soft fibrous inner surface of the tube.

15. The other references cited in the Office Action cannot be combined with Geistlich et al. and Shimizu to render the present claims obvious. Hentz et al. and Rosen et al. both disclose nerve repair wherein a membrane of hypoantigenic collagen (without a soft fibrous inner surface) is wrapped around a nerve. These references are not combinable with the other applied references to render obvious or make predictable the unexpected results achieved with the present invention. Stensaas et al. has no soft fibrous inner surface, and discloses a prosthesis for nerve regeneration which is made of a fluid-impermeable layer composed of silicone, rubber, polyurethane, Teflon or nitrocellulose. Stensaas et al. cannot be combined with the other applied references to render obvious, or make predictable, the unexpected results achieved with the present invention. Humes does not even relate to nerve regeneration tubes, but instead is directed toward a renal tubule tissue system wherein adult kidney cells are cultured in a medium which may contain Type I collagen and/or Type IV collagen. Humes cannot be combined with the other applied references to render obvious, or make predictable, the unexpected results achieved with the present invention.

16. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: ___October 18, 2007____ Signature: _____

Myron Spector, Ph.D.

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CURRICULUM VITAE

Myron Spector

Education

1967 B.S.	Carnegie Institute of Technology (Civil Engr.), Pittsburgh, PA
1969 M.S.	Carnegie-Mellon University (Civil Engr./Materials)
1971 Ph.D.	Carnegie-Mellon University (Civil Engr. - Biotechnology)

Postdoctoral Training (Research Fellowships)

1971	NIH Fellow, Biochemistry/Crystallography University of Pittsburgh
1972	NASA - American Society Engineering Education, Summer Faculty Fellow, Cellular Analytical Laboratory, L.B. Johnson Space Center, Houston
1973	NASA - American Society Engineering Education, Summer Faculty Fellow, Cellular Analytical Laboratory, L.B. Johnson Space Center

Current Academic Appointments:

Mar. 1993-present	Professor of Orthopedic Surgery (Biomaterials), Harvard Medical School
June 1993-present	Senior Lecturer, Department of Mechanical Engineering Massachusetts Institute of Technology
1991-present	Senior Lecturer, Harvard-MIT Division of Health Sciences and Technology

Current Hospital Appointments:

1987-present	Director of Orthopedic Research, Brigham and Women's Hospital
2002-present:	Director, Tissue Engineering, VA Boston Healthcare System, Boston, MA

Previous Academic Appointments:

1969-1970	Instructor of General Engineering, The Pennsylvania State University, New Kensington Campus
1972-1974	Assistant Professor of Biomedical and Civil Engineering, Clemson University
1974-1976	Assistant Professor of Biological and Physical Sciences and Pathology, Medical University of South Carolina
1976-1980	Associate Professor of Biological and Physical Sciences and Pathology, Medical University of South Carolina
1980-1982	Professor of Biological and Physical Sciences and Pathology, Medical University of South Carolina
1982-1987	Professor of Orthopaedics, Emory University School of Medicine
1982-1987	Associate Professor of Pathology, Emory University School of Medicine
1987-1992	Lecturer in Orthopedic Surgery, Harvard Medical School
1991-1993	Lecturer, Department of Mechanical Engineering Massachusetts Institute of Technology

Other Previous Professional Positions:

1968-1969	Senior Bioengineer, Westinghouse Research Laboratories, Pittsburgh
1982-1987	Affiliate Scientist, Emory University Yerkes Regional Primate Research Center

1982-1987	Principal Research Engineer, Department of Chemical Engineering, Georgia Institute of Technology
1982-1987	Director of Research, Department of Orthopaedics, Emory University School of Medicine
1987-2002	Director, Rehabilitation Engineering R&D Laboratory, Brockton/West Roxbury Veterans Administration Medical Center

Major Visiting Appointments:

1974	Visiting Professor of Neurology, Medical University of South Carolina
1987	Visiting Professor of Orthopedic Surgery (Basic Sciences), Mayo Clinic
1991	Hunter Visiting Professor of Bioengineering Clemson University (April 11-12, 1991)
1993	Ramon B. Gustilo Visiting Professor in Biomechanics Hennepin County Medical Center and University of Minnesota (January 22-23, 1993)
1995	Visiting Professor Military Postgraduate Medical School and Chinese PLA General Hospital, Beijing, China
1995	Visiting Professor Peking Union Medical Collage Hospital of the Chinese Academy of Medical Sciences, Beijing, China
1995	Visiting Professor Ninth People's Hospital, Shanghai Second medical University Shanghai, China
1995	Visiting Professor Beijing Ji Shui Tan Hospital, Beijing Institute of Traumatology and Orthopedics, Beijing, China
1998	Visiting Professor The First Military Medical University and Nanfang Hospital, Guangzhou, China

2001	Visiting Professor Taipei Medical University Taipei, Taiwan
2002	Visiting Professor of Orthopaedic Surgery West China Medical School of Sichuan University, Chengdu, China
2002	Visiting Professor Jiangsu University, Zhenjiang, China

Awards and Honors:

1967	American Society for Testing and Materials Student Award
1978-1983	Research Career Development Awardee, U.S. Public Health Service (NIH)
1991	The 10th Annual Hunter Honors Lecturer, Dept. of Bioengineering, Clemson University
1993	M.A. Harvard University (Honoris Causa)
1998-2001	Guest Professor of the Department of Materials Science and Engineering Tsinghua University, China
2001	Elizabeth Winston-Lanier Kappa Delta Award from the American Academy of Orthopaedic Surgeons/Orthopaedic Research Society for the work entitled, "Expression of Muscle Actin in and Contraction of Chondrocytes, Osteoblasts, and Musculoskeletal Tissue Fibroblasts." Highest research award given by these societies.
2002	Clemson Award for Applied Biomaterials Research. Highest award in this category given by the Society for Biomaterials.
2002-2007	Rehabilitation Research and Development Research Career Scientist Award from the U.S. Department of Veterans Affairs
2004	John Charnley Award for 2004 from the Hip Society for the work entitled, "The Role of Joint Fluid in the Tribology of Total Joint Arthroplasty." Highest research award given by this prestigious society.
2006-Present	Fellow, American Institute for Medical and Biological Engineering

Major Committee Assignments

National and Regional

1986-1990	NIH Orthopaedics and Musculoskeletal Study Section
1991-present	Food and Drug Administration General and Plastic Surgery Devices Advisory Panel (Chairman, 1993-1997) (Ad hoc Consultant, 1998-present)
1993-present	NIH Orthopaedics and Musculoskeletal Ad Hoc Reviewer
1993-present	Orthopaedic Research and Education Foundation Research Grants Peer Review Committee (Co-chairperson, 1995-1999)
1994-present	Food and Drug Administration Senior Biomedical Research Service Credentials Committee

Advisory Boards

1985-1995	NIH-DRR National Advisory Board, National ESCA and Surface Analysis Center for Biomedical Problems, University of Washington
2002-present	Committee Member of the Key Laboratory of the Ministry of Public Health of China
2004-present	Scientific Advisory Board, Institute for Bioengineering and Nanotechnology, A*STAR, Singapore

Journal Editorship

2005-present	Co-Editor-in Chief, Biomedical Materials: Materials for Tissue Engineering & Regenerative Medicine

Editorial Boards

1980-present	Editorial Board, Journal of Biomedical Materials Research
1982-present	Editorial Board, Biomaterials

1987-1992	Editorial Advisory Board, Journal of Orthopedic Research
1994-present	Editorial Board, Tissue Engineering
1996-present	Editorial Board Consultant, Chinese Journal of Arthroplasty
1998-present	Consultant Reviewer, Journal of Bone and Joint Surgery
1998-present	Editorial Board, Cell Transplantation
2003-present	Editorial Board, Orthopedics Today
2004-present	Editorial Board, Journal of Materials Science. Materials in Medicine
2007-present	Editorial Board, Frontiers of Materials Science in China

Memberships, Offices and Committee Assignments in Professional Societies

1971-present	American Association for the Advancement of Science
1973-present	Society for Biomaterials Council Member (1977-1982, 1988, 1989-present) Program Chairman (1982, 1989) President (1990-1991) Local Arrangements Chair (1994 Annual Meeting)
1973-1982	American Society for Testing and Materials
1978-present	Orthopaedic Research Society Local Arrangements Chairman (1988) Nominating Committee (2003)
1975-1982	International Association for Dental Research
1984-1995	American Society for Bone and Mineral Research
1989	Symposium Organizer, American Society of Mechanical Engineers
1989-1993	Academy of Surgical Research
1989-present	Materials Research Society Co-chairman Biomaterials Symposium, Nov., 1996
1989-present	American Academy of Orthopaedic Surgeons

1989-present	The Hip Society
1993-present	The Wound Healing Society
1995-present	Society for Tissue Engineering
1997-present	International Cartilage Repair Society Executive Board Symposium Organizing Committee, 1998
1996-present	Beijing-Shanghai-Boston Orthopaedic Education and Research Initiative Executive Board Symposium Organizing Committee, 1997, 1999

Major Research Interests

1. Tissue engineering: collagen analogs of extracellular matrix to be used as implants to facilitate regeneration of selected musculoskeletal and neural tissues spinal cord, including: articular cartilage, intervertebral disc, meniscus, ligament/tendon, peripheral nerve, spinal cord, and brain; investigations *in vitro* and *in vivo*. Work includes the investigation of the effects of mechanical loading on the cell-seeded construct and on reparative tissue, and the incorporation of plasmid DNA into the collagen scaffolds for non-viral gene therapy.

2. Expression of smooth muscle actin in and contraction of musculoskeletal connective tissue cells.

3. Biomaterials-tissue interactions. Healing of musculoskeletal tissues and the tissue response to implants (including the roles of endogenous and exogenous mechanical forces).

4. The role of joint fluid in the performance of joint replacement prostheses.

Current Teaching (The Massachusetts Institute of Technology and Harvard University)

1991-	Biomaterial-Tissue Interactions, 2.79J/3.96J/BEH.441HST 522J, MIT Fall Terms (Developed and teach this course with Prof. I. Yannas of MIT.)
1992-	Cell-Matrix Mechanics 2.785J/3.97J/20.411J/HST523J, MIT Spring Terms (alternate years) (Developed and teach this course with Prof. I. Yannas of MIT.)
1995-	Design of Medical Devices/Implants 2.782J/3.961J/20.451J/HST524J, MIT Spring Terms (Developed and teach this course with Prof. I. Yannas of MIT.)
2003-	Principles and Practice of Tissue Engineering, HST.535 Fall Terms (Developed this subject with Professor, F-Z. Cui of Tsinghua University, Beijing, China. The subject is taught with Tsinghua via a videoconference connection.)

Bibliography (per Harvard Medical School format)

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In: Johari O, ed. Scanning electron microscopy/1976. Chicago: IITRI Press, 1976:299.

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Histological Response to a Fully Degradable Collagen Device Implanted in a Gap in the Rat Sciatic Nerve

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ABSTRACT

Methods for engineering the regeneration of peripheral nerve in lesions have generally focused on the implementation of tubes as implants to bridge the defect. Previous study has shown that a highly porous analog of the extracellular matrix of a specific pore size range, ensheathed by a silicone tube, enhanced the regeneration of axons across gaps of 10 mm and greater in a transected adult rat sciatic nerve model. This study reports the histological findings resulting from implantation of a fully degradable collagen device comprising the collagen-glycosaminoglycan (GAG) analog in a collagen tube in a 10-mm gap in this animal model. Silicone tubes, with and without the collagen-GAG matrix, served as controls. Results indicated that axons had regrown into the midsection of the gap in all prostheses by 30 weeks; however, in the presence of the collagen-GAG matrix, the number and size of the axons appeared to increase. A layer of fibrous tissue approximately 100 μm thick, which contained fibroblasts, surrounded the silicone tubes but was not visible along the tube wall in any of the collagen tube prostheses. These findings show the promise of a fully degradable prosthesis for facilitating regeneration following peripheral nerve injuries.

INTRODUCTION

NERVE REGENERATION differs from the regenerative mechanism in connective tissue, muscle, and epithelia in that mitosis of the parenchymal cell is not required; in the nerve, regeneration depends on the elongation (regrowth) of cell processes (i.e., axons) destroyed by the injurious agent. Untreated gap lesions in peripheral nerve do not regenerate functioning tissue. Although axons in an untreated lesion elongate, suggesting the potential for regeneration, they have no guidance toward the distal stump and form a tangled web of unorganized axons, termed a *neuroma*.[1] In connective tissues and epithelia, autografts replace cells and

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extracellular matrix that have been damaged; in contrast, the peripheral nerve autograft serves as a guide for elongation of axons from the proximal nerve stump. This suggests that biomaterials may be used as synthetic substrates, in the same way that the autograft serves as a natural substrate. Silicone tube implants have been used as nerve guides to facilitate the *in vivo* regeneration of axons across gaps of 10 mm or less. In larger gaps of 15 to 20 mm, however, axons were not able to cross the gap when bridged by an empty silicone tube.[2] Bioresorbable tubes, including synthetic polymers,[3–6] tissues,[7–9] and naturally occurring polymers[10,11] have shown more potential for bridging nerve gaps and have the added benefit of being degradable.

Many investigators have studied, with some success, the effect of substrate materials, contained within tubes, on peripheral nerve regeneration. Examples of substrates used include the extracellular matrix proteins laminin, collagen, and fibronectin.[12–15] In particular, a collagen-glycosaminoglycan (GAG) matrix, ensheathed by a silicone tube, induced neovascularization and the regeneration of unmyelinated and myelinated axons across a 15-mm gap in the rat sciatic nerve.[16] Unfilled tubes yielded no more than trace numbers of axons or blood vessels within the gap. Further study of the collagen-GAG matrix contained in a silicone tube, using a 10-mm gap in the rat sciatic nerve, indicated that the pore structure and chemistry of the matrix influenced the success of regeneration. Small, axially aligned pores, 5 to 10 μm in diameter, and a specific molecular weight between cross-links of 60 kD, were found to lead to maximal regeneration.[17] This matrix was distinctly different from the collagen-GAG matrix that induced dermal regeneration in animals[18–20] and humans.[21,22] The collagen-GAG matrix optimal for dermal regeneration had similar chemical composition; however, the pores were randomly oriented with an average diameter of 20 to 125 μm and a molecular weight between cross-links of approximately 12 kD.[20]

The purpose of this study was to engineer peripheral nerve in lesions *in vivo* by developing a fully degradable device, comprising a collagen tube filled with a collagen-GAG matrix as a preferential substrate for axonal regrowth. The emphasis of this study was to examine the histological differences at the center of the nerve lesion between silicone and collagen tubes used as nerve guides.

MATERIALS AND METHODS

Adult female Lewis rats (Charles River Laboratories, Wilmington, MA), 175–200 grams, were used in this study. The sciatic nerve was transected midway between the proximal nerve trunk and the distal bifurcation using microscissors. Tubular prostheses (see below), either empty or filled with a collagen-GAG matrix, were placed in the gap. The proximal and distal nerve stumps were inserted 5 mm into each end, leaving a 10-mm gap. The nerve was secured in place using two 10-0 sutures (Ethicon) at each end (Fig. 1). No nerve tissue was removed during the procedure. Following transection, the nerve ends retracted approximately 5 mm and then were additionally separated to create the 10-mm gap. Some bending of the nerve occurred due to the added length, but no significant buckling was apparent. The 10-mm gap lesion was selected over the 15-mm gap lesion used in previous experiments for two reasons. First, the 15-mm gap requires a cross-anastomosis procedure to the contralateral side due to lack of space in the femoral re-

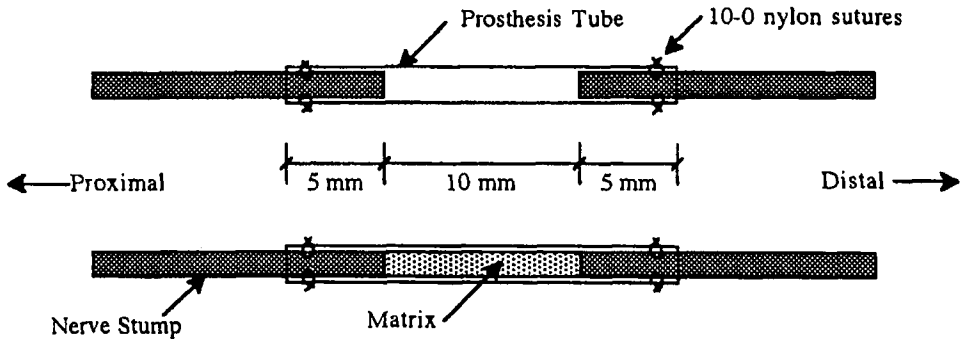

FIG. 1. Diagram showing an empty prosthesis tube (top) and a collagen-GAG matrix-filled tube (bottom) after surgical implantation in the rat sciatic nerve.

gion of the rat. This type of lesion results in more complex animal care since both sciatic nerves are injured. Second, the 10-mm gap lesion is commonly studied, and therefore more data are available for comparison. The animals were monitored daily for the appearance of any abnormal behavior, such as insufficient grooming, autotomy, lack of appetite, and aggressive behavior.

Several prostheses were evaluated: porous collagen and nonporous collagen tubes (Integra Life Sciences, Plainsboro, NJ) were implanted, both empty and filled with a collagen-GAG matrix. Both tube types were made from type I tendon collagen and had an inside diameter of 1.5 mm. The maximum pore diameter of the porous collagen tubes was 22 nm,[11] and for the nonporous tubes it was 4 nm.[23] Control devices included a silicone tube, with a 1.5-mm inside diameter (Silastic Medical Grade Tubing, Cat# 602-235, Dow-Corning Co., Midland, MI), implanted both empty and filled with the collagen-GAG matrix.

The collagen-GAG matrix was prepared as previously described.[17,24] Type I bovine hide collagen in acetic acid solution was coprecipitated with chondroitin 6-sulfate. A suspension of the precipitate was injected into silicone processing tubes, and the tubes were frozen. The frozen, slurry-filled tubes were placed in a freeze dryer and lyophilized to produce a highly porous matrix with axially aligned pores. The matrices were sterilized and cross-linked by dehydrothermal treatment.[25] Specimens of the collagen tubes and the collagen-GAG matrix were prepared for the environmental scanning electron microscope.

In this study, 35 animals were sacrificed after 30 weeks of implantation, using a transcardial perfusion by mixed aldehydes. The sciatic nerve was explanted from the sciatic notch at the hip to beyond the bifurcation point at the knee level, including portions of the tibial and peroneal nerve branches. Specimens of normal sciatic nerve were obtained as anatomic controls. Tissues were processed and embedded in either Epon plastic or paraffin according to standard methods. Microtomed paraffin sections were stained with hematoxylin and eosin and Masson's trichrome. Epon samples, sectioned on an ultramicrotome to a 1-μm thickness, were stained with toluidine blue.

Cross-section areas of the collagen tubes and regenerated tissue, at the midportion of the gap, were measured from low magnification images that were captured into a Macintosh computer using a video camera connected to the light microscope. Measurements were obtained using image analysis software (NIH Image, public domain software). The tube wall cross-section area was calculated by subtracting the luminal area, measured from the tube inner diameter, from the total cross-section area, measured from the tube outer diameter. A random sampling of high magnification images was taken for each nerve, and the number of axons was counted using image analysis.

RESULTS

Morphological examination of the collagen-GAG matrices employed in this study revealed axially aligned pore channels, approximately 20–50 μm in diameter (Fig. 2A). Within and around each large pore channel, there was a meshwork structure consisting of thin collagen filaments (100–1000 nm thick) that intersected frequently (Fig. 2B). In many cases, the collagen filaments bridged the pore channels (Fig. 2A). When viewed in cross-section, the network of collagen filaments produced pores on the order of 10 μm (Fig. 2B). The porous collagen tubes (Fig. 2C) had collagen layers, arranged in a laminar fashion, that were loosely packed. The walls of the porous collagen tubes were approximately 650 μm thick. In contrast, the nonporous collagen tubes (Fig. 2D) had a more compact arrangement of collagen and a wall thickness of approximately 80 μm. In both collagen tubes, the luminal surfaces were smooth.

The general condition of the animals was good throughout the duration of the experiment. The animals adjusted well to the nerve lesion and showed no signs of severe discomfort. They had sufficient mobility to eat and drink as normal, even in the early weeks following surgery. There were no signs of lost appetite or lack of grooming at any time during the experiment. One exception was that the rats in the experimental group did not trim their toenails. This is a common finding after sciatic nerve transection.[26] Throughout the current experiment, none of the animals expressed any degree of autotomy. Flexion toe contractures on the operated limb were observed in most of the animals, beginning at 6 weeks. Some of the animals also developed heel ulcers, another common deformity following sciatic nerve transection.[26] Atrophy of the muscle of the operated limb was visible in some animals as early as 6 weeks postoperative.

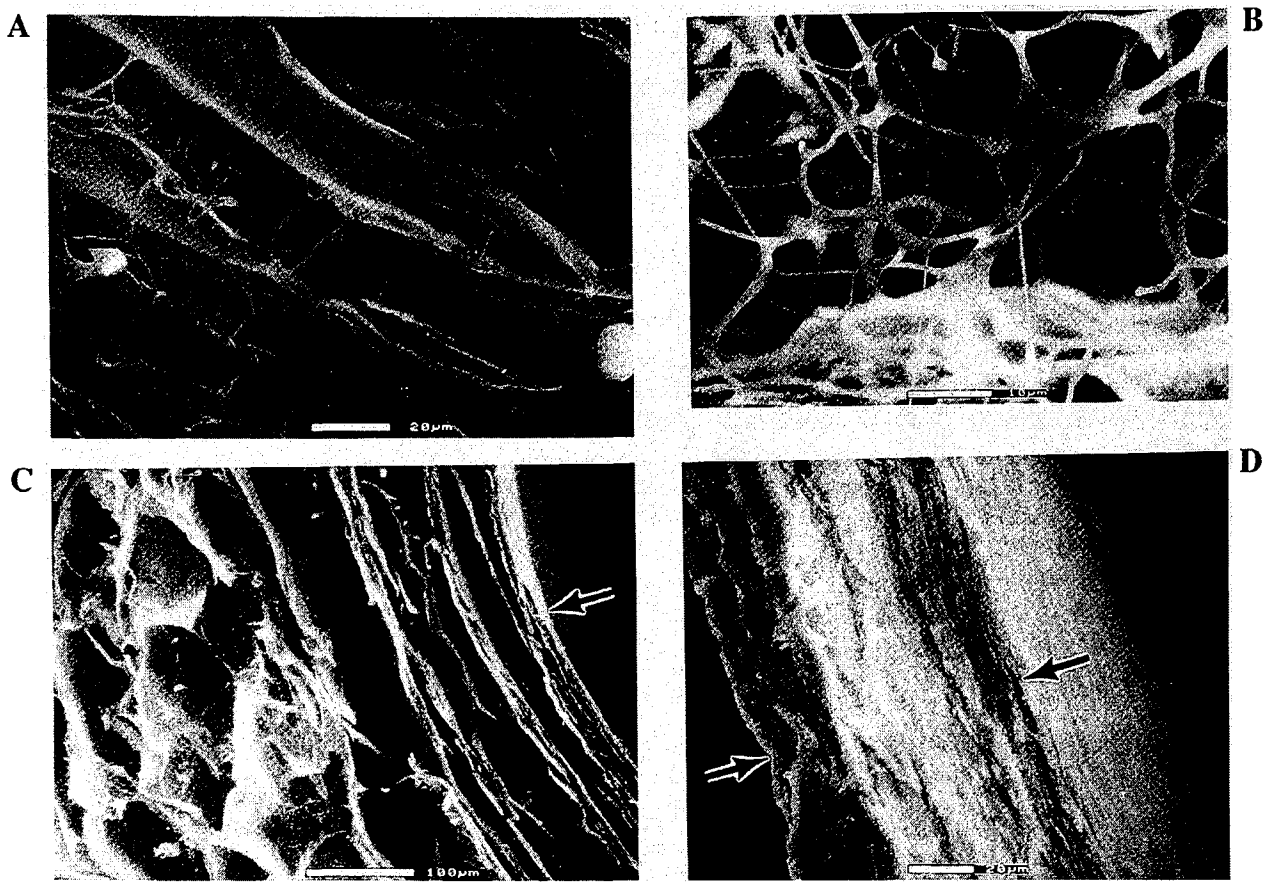

FIG. 2. Environmental scanning electron micrographs of the collagen-GAG matrix and collagen tubes. (A) Longitudinal view of the collagen-GAG matrix that has pore channels, approximately 20–50 μm in diameter, oriented parallel to the nerve axis. The nerve axis in this micrograph is from the upper left to the lower right corner. Note the collagen filaments, perpendicular to the nerve axis, bridging the pore channels. (B) Cross-section view of the meshwork of collagen filaments located within and around each pore channel. The mesh has pores with diameters on the order of 10 μm. (C) The porous collagen tube has loosely paked layers of collagen in a laminar arrangement. The tube wall thickness, measured from the luminal surface (arrow) to the outer surface (not visible), is approximately 650 μm. (D) The nonporous collagen tube has densely packed collagen layers and a wall thickness of approximately 80 μm. The luminal and outer surfaces are visible in this micrograph (arrows).

The transparency of the silicone tubes and the nonporous collagen tubes enabled visualization of the reparative tissue bridging the lesion at explantation. Gross observation of the explanted and dissected nerves revealed a tissue bridge connecting the proximal and distal segments in all of the collagen tube groups. The tissue completely filled and appeared to be adherent to the tubes (Fig. 3A). In the matrix-filled silicone tube group, five of six animals formed a tissue bridge that, unlike the collagen tube groups, filled only half the cross-section area (Fig. 3B). In the one animal in which reconnection did not occur, there were clinical signs of infection. In the empty silicone tube group, reconnection occurred in only three of six animals. Moreover, the tissue in the gap, when present, was a thin cable that filled less than 10% of the tube cross-section (Fig. 3C). The reparative tissue was not adherent to the silicone tubes. At this 30-week postoperative period, the collagen tubes, both porous and nonporous, were not noticeably degraded; the tubes appeared grossly to be about the same size as they were at implantation. The porous collagen tubes retained their original shape, but the nonporous collagen tubes partially collapsed, taking on an elliptical shape.

Histology of the reparative tissue in all of the tubes generally revealed few acute inflammatory cells such as polymorphonuclear neutrophils, eosinophils, and basophils; in some cases, macrophages were apparent near the tube surface. Inflammatory cells—lymphocytes and plasma cells—and excessive vascularity that may have signaled immunologic rejection of the bovine-derived implants were not present. The cells pres-

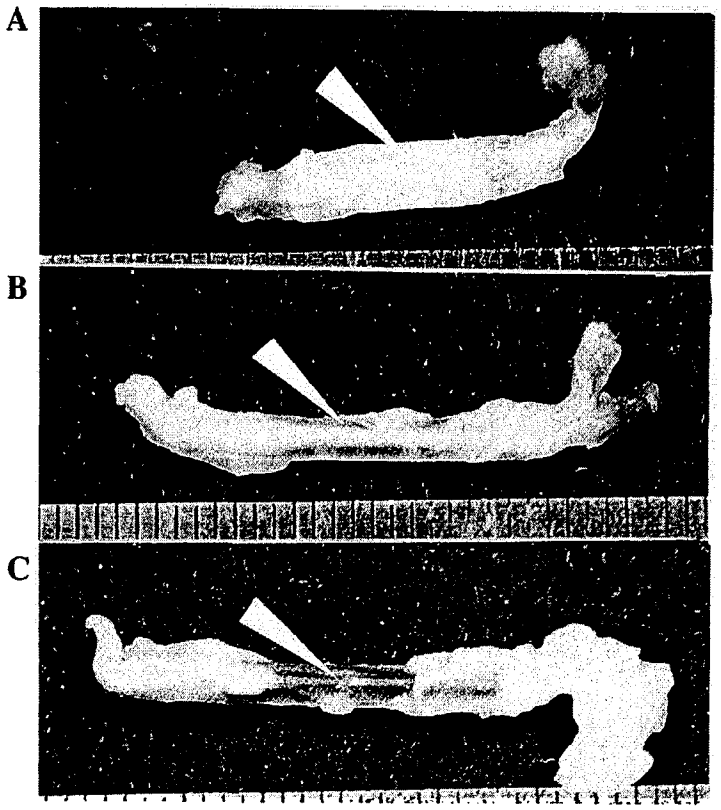

FIG. 3. Gross morphology of the regenerated nerves (arrows) after 30 weeks of implantation. (A) Matrix-filled nonporous collagen tube implant. The tube has a tissue cable connecting the proximal and distal stumps. The proximal nerve stump is on the left, and the distal nerve branches are visible on the right end of the tube. (B) Matrix-filled silicone tube implant. Note the thick tissue cable within the tube. (C) Empty silicone tube implant. The tissue cable is thin. The distal stump (on the right) is surrounded by fat, making the branches difficult to see.

ent within the defect site were primarily Schwann cells, associated with specific axons (Fig. 4B). In all groups, the regenerated tissue had increased vascularity compared to normal nerve tissue.

The collagen tubes, both porous and nonporous, generally appeared to be intact and undegraded at 30 weeks (Figs. 5A and 5B). Microscopic examination of the porous collagen tubes revealed separations between laminae comprising the wall of the tube, which were consistent with the tube structure preimplantation (Figs. 2C and 5A). Few cells were found within these larger pores in the walls of the porous collagen tubes or on the inner and outer surfaces of the tubes. One exception was the finding of fat cells in the outer pores of the porous collagen tubes (Fig. 5A). By 30 weeks, the tube wall cross-section area of the porous collagen tubes was reduced by 15% compared to preimplantation values. In contrast, the nonporous collagen tubes had a significantly different structure after 30 weeks of implantation (Figs. 2D and 5B). The collagen contained within the tube wall expanded when placed in the *in vivo* environment. No cells or axons were found within the walls of the tubes, suggesting that the tube expansion occurred soon after implantation (Fig. 5B), and few cells could be found on the tube surfaces. The nonporous collagen tubes had a 240% increase in the tube wall cross-section area, due to the expansion of the tube material. The inert and highly flexible nature of the silicone tubes prevented any deformation of the tubes. In contrast to both of the collagen tubes, the silicone tubes had fibrous tissue, containing many fibroblasts, arranged circumferentially around the tube (Fig. 5C). The fibrous capsule was approximately 100 microns thick and was directly apposed to the tube on the outer surface (Fig. 5C). Inside the silicone tube, a similar fibrous capsule surrounded the nerve tissue, which filled only approximately half the diameter of the lumen.

Masson's trichrome staining confirmed the presence of newly synthesized collagen within the defect site of all groups. The collagen orientation appeared random when viewed in cross-section. The fibrous tissue

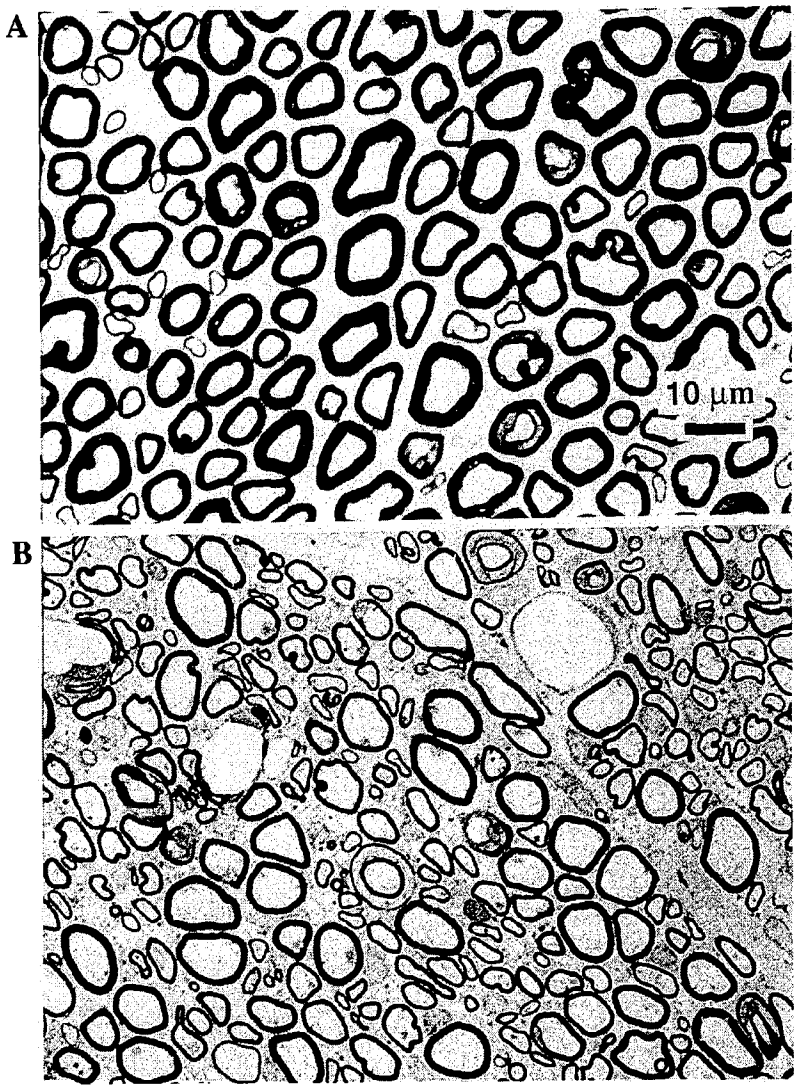

FIG. 4. Histological micrographs of peripheral nerve sections stained with 1% osmium tetroxide and toluidine blue and viewed at 100× magnification. Scale bar = 10 μm. (A) Normal nerve and (B) regenerated nerve (matrix-filled nonporous collagen prosthesis). Note the large axons, approaching 10 μm in diameter. Many small-diameter axons are present compared to the normal. Many Schwann cells are visible in the regenerated case.

appeared loosely packed, surrounding the axons, and was more prominent than in normal nerve tissue. By 30 weeks, no residual collagen-GAG matrix was visible. Prior to implantation, each tube had the same luminal area (1.8 mm^2); however, due to the structural changes of the collagen tubes *in vivo*, the luminal area in each case was reduced. In the collagen tubes, the trichrome stain confirmed that the tissue completely filled the tubes at 30 weeks; consequently, the luminal and tissue areas were equal. In the silicone tubes, the luminal area was unchanged after 30 weeks of implantation, but the tissue cables did not fill the tubes, with the balance being filled with fluid exudate and a semisolid gel-like material. The matrix-filled silicone and nonporous collagen tubes had tissue areas (0.4 ± 0.05 mm^2 for each) comparable to normal (0.5 ± 0.05 mm^2). In contrast, the porous collagen tubes, both empty and matrix filled, had tissue areas (1.4 ± 0.1 mm^2 and 1.3 ± 0.1 mm^2, respectively) nearly three times larger than normal. The remaining implant groups—empty silicone and nonporous collagen—had tissue areas (0.1 ± 0.05 mm^2 and 0.3 ± 0.05 mm^2, respectively) smaller than normal.

Axons were found to have elongated into the center of the defect in every device where tissue was present (Fig. 4B). In many cases, the axons were bundled into groups of 10–100 axons, which previously have

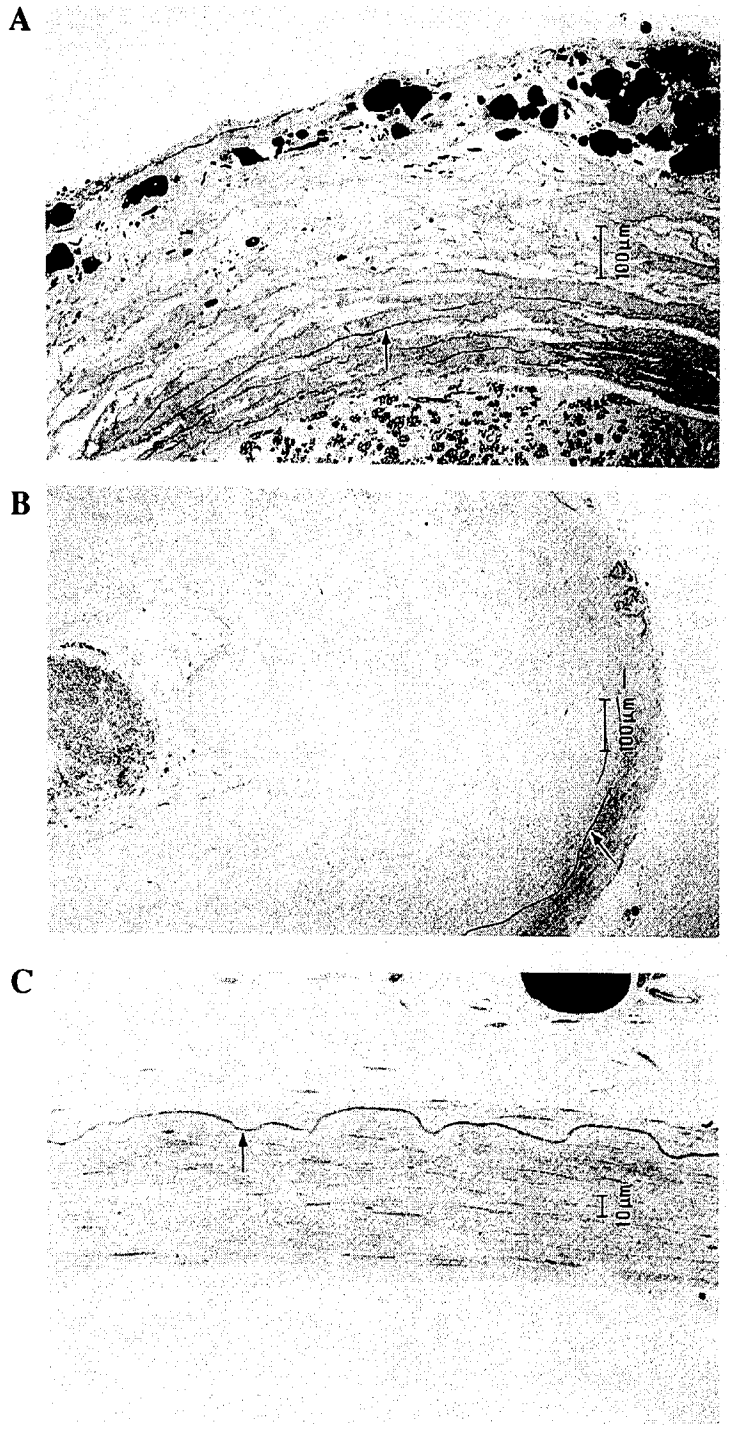

FIG. 5. Histological micrographs of the (A) porous and (B) nonporous collagen tubes and the (C) fibrous tissue surrounding the silicone tube following 30 weeks of implantation in the rat sciatic nerve. Sections were stained with 1% osmium tetroxide and toluidine blue. In each micrograph, arrows identify wrinkles in the samples. (A) Fat cells are apparent in some of the outer laminae of the porous collagen tube. This laminar arrangement is consistent with the structure of the tube preimplantation. The tube wall thickness is approximately 600 μm, compared to 650 μm preimplantation. (B) The walls of the nonporous collagen tube have expanded significantly, to a thickness of 900 μm in this case, compared to an 80-μm thickness preimplantation. No cells or axons are present within the walls of the tube. (C) This fibrous capsule, found on the outer surface of the silicone tube, is approximately 100 μm thick and contains elongated fibroblasts.

been termed *minifascicles*.[27] The axon bundles were usually surrounded by a ring of collagenous tissue. In the collagen tubes, this fibrous tissue containing the minifascicles was adherent to the tubes. The regenerated nerve tissue in all groups appeared to have a density of axons equal to or higher than normal nerve (Figs. 4A and 4B), and there was more connective tissue surrounding the axons than normal. In part, this was due to the fact that the axons were smaller in diameter than normal, with thin myelin sheaths. Some large-diameter axons were visible, especially in tubes that contained the collagen-GAG matrix. Each group, except the empty silicone devices that contained about 2,500 axons, had more myelinated axons than normal nerve (6,000 axons). The implant groups that had tissue areas similar to normal—matrix-filled silicone and nonporous collagen—had nearly twice as many axons packed in the same amount of space, with myelinated axon counts ranging from 9,000 to 12,000 axons. The porous collagen tubes, which had significantly more space for axons to occupy, had axon counts that fell into a similar range (11,000 to 16,000 axons). In addition, the matrix-filled tube groups (9,000 to 16,000 axons) had more myelinated axons, in general, than the empty tube groups (2,500 to 11,000 axons).

DISCUSSION

The rats were monitored daily for any signs of autotomy, which was a major concern during this study because of excessive autotomy during a previous experiment using Sprague-Dawley rats.[24] None of the animals in the current study displayed autotomy, which confirmed reports that the Lewis strain of rats had no inclination toward self-mutilation[28] and therefore is a good choice for studies involving sciatic nerve transection.

The volume of reparative tissue in the silicone and collagen tubes was notably different, with tissue in the collagen tubes filling the entire gap volume and tissue in the silicone tubes filling less than half of the lumen of the tube. Moreover, a thick layer of fibrous tissue, containing elongated fibroblasts, was found along the inner and outer aspects of the silicone tubes at 30 weeks. In a previous study, which employed the same tubes, contractile cells (myofibroblasts) were identified at 6 weeks postoperation in a similar fibrous tissue layer that encapsulated the silicone tubes; however, few myofibroblasts were identified in conjunction with the collagen tube surfaces.[29] Therefore, although the collagen tubes remained undegraded at 30 weeks, they did not display, at an earlier time period, characteristics (i.e., a thick fibrous layer with contractile cells) that may lead to entrapment or constriction following long-term entubulation. Clinically, long-term silicone entubulation has resulted in pain, caused by chronic nerve compression, that eventually led to surgical removal of the silicone tubes.[30] These results suggest that collagen may be a superior biomaterial for the purpose of fabricating tubes for bridging peripheral nerve lesions.

The increase in the thickness of the tube wall in the nonporous collagen tubes was significant and reduced the luminal area of the tube. Similar findings have been reported with resorbable polyester tubes that, in some cases, had swelled enough to block the lumen completely.[31] These results suggest that degradable tubes must be sufficiently cross-linked to prevent early swelling of the tube material resulting in closure of the lumen. In contrast, the porous collagen tubes had a reduction in tube wall cross-section area of 15% by 30 weeks postoperation that could have been due to degradation or the result of the laminae compacting within the tube wall (Fig. 5A). The porous collagen tubes that displayed little degradation at 30 weeks in our study have been shown in previous investigations in a primate model to have almost completely resorbed by 2 years;[11] complete resorption occurred by 3.5 years.[10]

The finding that tissue bridged the gap in only half of the empty silicone tubes is surprising since many investigations using this implant for a 10-mm gap have found bridging in nearly all cases.[2,32,33] The inconsistent bridging in our experiment may have been due to the strain of rat used (each of the aforementioned studies used Sprague-Dawley rats) or variations in the surgical procedure between investigators. Each animal in this study received the same treatment, and the surgery was performed in a consistent manner, by the same individual. Although our empty silicone tube results differed from previous use of this implant, this group was used only as a control in this study for comparisons among prostheses.

Axons were present in all groups, but they appeared in greater numbers in tubes filled with collagen-GAG matrix, confirming previous studies.[16,17] The effect of the collagen-GAG matrix was most apparent

in the silicone tube case, where the tissue area and total number of axons were significantly increased in the presence of the matrix. There was no correlation between the number of axons and the luminal and tissue areas. For example, the porous collagen tube groups had approximately three times the tissue area as the matrix-filled silicone and nonporous collagen groups, but the numbers of axons were within comparable ranges. Maturation of the axons was evident based on the presence of some larger diameter axons, which appeared more prominent in the matrix-filled tubes. Based on the large number of axons, it appears that significant sprouting of axons has occurred in each of the regenerated nerves. Longer term study will be required to determine the extent to which the regenerating nerves achieve normal numbers and size distributions of axons. Moreover, functional tests may be used in future studies to assess the degree to which the axons have made connections with distal targets.

Histological differences exist between prostheses comprising silicone and collagen tubes. Differences included the volume of reparative nerve tissue, the amount and type of fibrous tissue surrounding the tube, and tissue adherence to the tube, with the collagen tubes yielding more favorable results. This finding, coupled with the resorbability of collagen tubes, indicates that collagen tubes are superior to silicone tubes for bridging nerve gap lesions.

ACKNOWLEDGMENT

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Connective Tissue Response to Tubular Implants for Peripheral Nerve Regeneration: The Role of Myofibroblasts

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ABSTRACT

The presence of contractile cells, their organization around regenerating nerve trunks, and the hypothetical effect of these organized structures on the extent of regeneration across a tubulated 10-mm gap in the rat sciatic nerve were investigated. Collagen and silicone tubes were implanted both empty and filled with a collagen-glycosaminoglycan (GAG) matrix. Nerves were retrieved at 6, 30, and 60 weeks postoperatively and time-dependent values of the nerve trunk diameter along the tubulated length were recorded. The presence of myofibroblasts was identified immunohistochemically using a monoclonal antibody to α-smooth muscle actin. Myofibroblasts were circumferentially arranged around the perimeter of regenerated nerve trunks, forming a capsule which was about 10 times thicker in silicone tubes than in collagen tubes. The nerve trunk diameter that formed inside collagen tubes was twice as large as that inside silicone tubes. In contrast, the collagen-GAG matrix had a relatively small effect on capsule thickness or diameter of regenerate. It was hypothesized that the frequency of successful bridging by axons depends on the balance between two competitive forces: the axial forces generated by the outgrowth of axons and nonneuronal cells from the proximal stump and the constrictive, circumferential forces imposed by the contractile tissue capsule that promote closure of the wounded stumps and prevent axon elongation. Because the presence of the collagen-GAG matrix has enhanced greatly the recovery of normal function of regenerates in silicone tubes, it was hypothesized that it accelerated axonal elongation sufficiently before the hypothetical forces constricting the nerve trunk in silicone tubes became sufficiently large. The combined data suggest a new mechanism for peripheral nerve regeneration along a tubulated gap. J. Comp. Neurol. 417: 415–430, 2000.

Indexing terms: peripheral nerve; regeneration; collagen; myofibroblasts; connective tissue; neuroma, silicone; contraction; collagen-GAG matrix

In a widely used model for the study of peripheral nerve regeneration, the stumps of the transected nerve are secured inside a tube (tubulation) that is used to bridge a gap of known length (Lundborg, 1987; Fu and Gordon, 1997). Neuroma formation occurs unless axons can elongate from the proximal stump and extend across the tubulated gap to reach inside the distal stump (Dellon, 1990). Implantation of a tubular device in the wound site reduces the likelihood of neuroma formation (Fields et al., 1989). Several theories have been proposed to account for the successful use of tubulation in inducing regeneration across transected gaps in the peripheral nervous system (see reviews by Lundborg, 1987; Fu and Gordon, 1997;

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Valentini and Aebischer, 1997). However, tubulation does not always prevent neuroma formation. For example, use of a silicone tube leads to regeneration across gaps of 10 mm or less in the rat sciatic nerve; however, an increase in gap length to 15 mm or longer results in neuroma formation in all cases (Lundborg et al., 1982a, 1997; Williams et al., 1983; Yannas et al., 1987).

The transected peripheral nerve is subject to wound healing processes largely similar to those observed in other organs, such as skin (Dellon, 1990). Wound contraction is an important part of skin wound healing because it brings wound edges into approximation and facilitates wound closure. Myofibroblasts have been identified as important participants in the contraction of healing wounds in the skin (Gabbiani et al., 1971) and in several other organs (Desmoulière and Gabbiani, 1996; see review by Gabbiani, 1998). In particular, myofibroblasts have recently been identified in the regenerating peripheral nerve after 6 weeks (Chamberlain, 1996; Chamberlain et al., 1998a). The role of the myofibroblast in peripheral nerve wound healing will be addressed further in this study.

The primary objective of this investigation was to study the connective tissue of the adult rat sciatic nerve that formed in response to two tubular implant parameters: tube composition (collagen vs. silicone) and the presence of a collagen-glycosaminoglycan (GAG) matrix with a highly specific structure, which has been shown to be capable of encouraging axon elongation over long distances in tubulated gaps (Yannas et al., 1987; Chang et al., 1990; Chang and Yannas, 1992). Untreated animals as well as intact, contralateral nerves were included as controls. The nerve trunk diameter and the tissue capsule thickness at the perimeter of the trunk were studied at 6, 30, and 60 weeks postoperatively. The presence of contractile cells (i.e., myofibroblasts) in the tissue capsule, nerve trunk, and tube walls was determined immunohistochemically by using a monoclonal antibody against the α-smooth muscle actin isoform.

This appears to be the first systematic evaluation of the presence of myofibroblasts in the tubular implants in defects in peripheral nerve. Our earlier transmission electron microscopy study reported a finding of myofibroblasts in reparative tissue adjacent to a silicone tube implant after 6 weeks (Chamberlain et al., 1998a); the present study extends the study to 60 weeks. We report the effects of tube composition and the presence of the collagen-GAG matrix as well as provide a new interpretation of the mechanism of peripheral nerve regeneration along tubulated gaps. The recent identification of myofibroblasts in healing nerve wounds (Chamberlain et al., 1997, 1998a, 1999) relates to persistent independent reports of concentric sheaths of fibroblast-like cells around the fibrin cable that form spontaneously inside the tubulated gap of the rat sciatic nerve (Lundborg et al., 1982b; Williams et al., 1983; Williams and Varon, 1985; Jenq and Coggeshall, 1985a,b; Hurtado et al., 1987; Madison et al., 1988; Kljavin and Madison, 1991). It now appears to us that these cell structures should be designated as myofibroblasts and their role in wound healing in the peripheral nervous system (PNS) assessed accordingly.

MATERIALS AND METHODS

Peripheral nerve devices

The collagen tubes were fabricated from type I bovine tendon collagen and had an inside diameter of 1.5 mm (Integra LifeSciences, Plainsboro, NJ). Details of the procedure for molding collagen tubes have been described (Li et al., 1990, 1992; Archibald et al., 1991, 1995). These tubes have been reported to have walls with a maximum pore diameter of 22 nm, a size that excludes transfer of proteins with a molecular weight higher than 540 kD (Li et al., 1992). They were cross-linked with a gaseous formaldehyde treatment in order to decrease the degradation rate following implantation (Archibald et al., 1995). The silicone tubes (Silastic Medical Grade Tubing, Dow-Corning Co., Midland, MI) had an inside diameter of 1.5 mm. All tubes were sterilized at 105°C for 24 hours under vacuum. The sterilization process does not affect the periodicities either of the banding period or of the triple helix of collagen (Yannas, 1972).

The collagen-GAG matrix copolymer was prepared as previously described (Loree et al., 1989; Chang et al., 1990); it was similar in chemical composition but distinctly different in average pore diameter, orientation of pore channel axes, and degradation rate from a CG matrix, which has been shown capable of inducing regeneration of the dermis in a guinea pig model (Yannas et al., 1989). The collagen-GAG matrix comprised fibrous, type I bovine hide collagen (USDA, Philadelphia, PA) and chondroitin 6-sulfate (Sigma Chemical Company, St. Louis, MO) in a 98/2 wt/wt ratio. To prepare the matrix, a collagen-GAG suspension (collagen, chondroitin 6-sulfate, and acetic acid) was injected into silicone processing tubes and frozen under conditions that produced nearly optimal pore characteristics of the matrix, as determined by a 40-week electrophysiological study of several structurally related collagen-GAG matrices in silicone tubes (Chang et al., 1990; Chang and Yannas, 1992). Sublimation of ice crystals by freeze-drying left inside the silicone tube a cylinder of highly porous matrix (pore volume fraction of 0.95) with axially oriented pore channels, averaging 35 μm in diameter. The matrices were transferred to a 105°C oven under vacuum (Fisher Isotemp Vacuum Oven, Fisher Scientific, Boston, MA; VacTorr 150 Vacuum Pump, GCA/Precision Scientific, Chicago, IL) for cross-linking and sterilization (Yannas and Tobolsky, 1967; Yannas et al., 1989). This treatment leaves the triple helical structure of collagen intact, provided that the moisture content of collagen prior to heat treatment is less than 5 wt% (Yannas, 1972; Yannas et al., 1989). The resultant matrix had a half-life of approximately 6 weeks in the rat sciatic nerve.

In a sterile hood, the silicone and collagen tubes were trimmed into 20-mm lengths. If the prosthesis was matrix-filled, a 10-mm segment of collagen-GAG matrix was inserted into the tube. Each implant was placed in sterile phosphate-buffered saline (PBS; Sigma), pH 7.4, prior to implantation, and was considered to be at least partly filled with saline when implanted.

Animal model

Twelve adult female Sprague-Dawley rats, 200–230 g, and 40 adult female Lewis rats (Charles River Laboratories, Wilmington, MA), 175–200 g, were used in this study. The Sprague-Dawley rats were used in the initial short-term study (6 weeks) and a significant amount of autot-

omy, or self-mutilation, was observed. Therefore, the Lewis strain of rat was used for the long-term endpoints (30 and 60 weeks) because of its resistance to autotomy following sciatic nerve transection (Carr et al., 1992; Chamberlain et al., 1997). The animals were divided into five experimental groups, as follows: silicone tube (SI; n = 12), silicone tube filled with collagen-GAG matrix (SI/M; n = 12), collagen tube (LC; n = 12), collagen tube filled with collagen-GAG matrix (LC/M; n = 12), and no treatment (n = 4). The LC tubes were identical to tubes with the same designation that were studied recently in these laboratories (Chamberlain et al., 1998a,b).

The following protocol was approved by the Animal Care Committee of the West Roxbury Veterans Administration Medical Center and conforms to NIH guidelines. Each animal was anesthetized using an intraperitoneal injection of 50 mg/kg sodium pentobarbital (50 mg/ml Nembutal Sodium Solution, Parke-Davis, Ann Arbor, MI). Once the animal was fully anesthetized, the surgical area was shaved with animal clippers and cleaned using an iodine sponge. The animal was placed in the prone position on the surgical board, arms and legs secured, with the legs in 30° abduction. A 4-cm incision was made parallel to and just posterior of the femur. The sciatic nerve was exposed and further anesthetized topically using a few drops of 1% lidocaine placed directly on the nerve. The fascia surrounding the nerve was cut away so that the nerve was completely free from constraint.

For a tubular repair, the nerve was transected midway between the sciatic notch and the distal bifurcation using microscissors. The tubular prosthesis, either empty or filled with the collagen-GAG matrix, was placed in the gap and the proximal and distal nerve stumps inserted 5 mm into each end of the tube, leaving a gap, 10 ± 1 mm long, in the center. The nerve was secured in place using two 10-0 sutures (Ethicon, Somerville, NJ) at each end. For an animal receiving no treatment, a 5-mm segment of the sciatic nerve was removed from the midpoint between the sciatic notch and the distal bifurcation using microscissors. Removal of the 5-mm nerve segment and subsequent retraction of the nerve stumps resulted in formation of a 10-mm gap between the nerve stumps. The proximal and distal nerve stumps were each allowed to fall naturally onto the underlying tissue and no repair methods were employed. The muscle and skin were closed using 4-0 sutures and skin staples as needed. The animals were placed back in their cages and were monitored until fully alert. They were housed on wood chip bedding, two animals per cage, for the remainder of the experiment. Food and water were available ad libitum. The animals were monitored daily for signs of any abnormal behavior, such as insufficient grooming, lack of appetite, aggressive behavior, and the appearance of autotomy.

In each of the four tubulated groups, three animals at 6 weeks and six animals at 30 weeks postoperatively were sacrificed by transcardial perfusion with mixed aldehydes and the sciatic nerve tissue was prepared for histomorphometric analysis. At 60 weeks postoperatively, the remaining three animals in each tubulated group were sacrificed by intraperitoneal injection with an overdose of sodium pentobarbital and the tissue was processed for histomorphometric analysis. The animals receiving no treatment (n = 4) were sacrificed 6 weeks postoperatively, a time period long enough for sufficient formation of a neuroma.

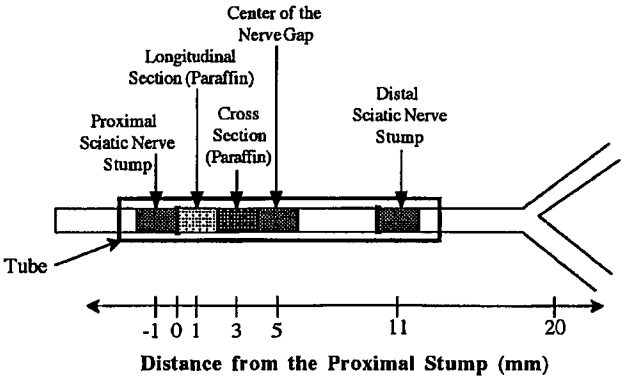

Fig. 1. Schematic outlining the tissue allocation for this study. Tissue sections from the proximal sciatic nerve stump, center of the nerve gap, and distal sciatic nerve stump were embedded in cross-sectional orientation in Epon and used to evaluate the nerve trunk diameter and tissue capsule thickness. A nerve segment, centered around 1 mm from the proximal stump, was embedded in longitudinal orientation in paraffin and used to evaluate the presence of axially aligned contractile cells in the nerve trunk. A nerve segment, centered around 3 mm from the proximal stump, was embedded in cross-sectional orientation in paraffin. This section was used to evaluate the presence of contractile cells in the tissue capsule, nerve trunk, as well as within and around the tube walls.

Tissue allocation

Following sacrifice at 6, 30, and 60 weeks postoperatively, the sciatic nerves regenerated through tubular devices were explanted from the sciatic notch at the hip to beyond the distal bifurcation point at the knee level. After explantation, the tissue was placed in Yanoff's fixative and sectioned into 2-mm segments, of which several were selected for analysis of the connective tissue response to nerve injury (Fig. 1). Two tissue segments from the proximal half of the nerve gap were retrieved and designated for paraffin embedding in either cross-section or longitudinal section (Fig. 1). These two tissue segments were embedded in paraffin (VWR Scientific, Boston, MA), cut on a microtome at an 8-μm thickness, and used for histological and immunohistochemical analysis. Additional tissue segments from the proximal nerve stump, center of the nerve gap, and distal nerve stump (Fig. 1) were postfixed in 1% osmium tetroxide (Polysciences, Inc., Warrington, PA) and embedded in Epon (Polysciences). The Epon-embedded sections were used to evaluate the nerve trunk diameter and the tissue capsule thickness.

Following 6 weeks, the neuromas resulting from no treatment were retrieved and placed in Yanoff's fixative. The entire proximal neuroma (n = 2) and the entire distal tissue (n = 2) were embedded in paraffin in longitudinal section. The remaining proximal (n = 2) and distal (n = 2) tissues were embedded in paraffin in cross-section. The specimens were sectioned at an 8-μm thickness and used for histological and immunohistochemical analysis.

Histology

Sections embedded in paraffin were stained with hematoxylin and eosin as well as with Masson's trichrome according to standard protocols. The hematoxylin and eosin stain was used to identify cells and Masson's trichrome stain was used to determine collagen organization within

the tissue. Epon samples were prepared by sectioning the samples on an ultramicrotome at a 1-μm thickness. The slides were additionally stained with toluidine blue (Fisher Biotech, Boston, MA) to enhance the color of the osmium stain. They were then mounted and coverslipped (Cytoseal 60 Mounting Medium, Stephens Scientific, Riverdale, NJ). Nerve trunk diameters were determined using digitized images and image analysis software (NIH Image). The tissue capsule thickness was measured using a scaled reticule on the light microscope. It was defined by the following characteristics: circumferential orientation of cells and connective tissue, located around the perimeter of the nerve trunk (in cross-section), and contained no axons.

Immunohistochemistry

The immunohistochemical technique was intended to stain the α-smooth muscle actin isoform, a distinguishing characteristic of myofibroblasts (Gabbiani, 1998). Paraffin sections were cut to an 8-μm thickness using a microtome and placed on glass slides. The sections were deparaffinized and then treated with a 0.1% trypsin solution (Sigma) for 60 minutes at room temperature to unmask antigen sites blocked by formalin fixation. The slides were quenched with a 3% hydrogen peroxide solution, followed by 20% nonimmune goat serum blocking solution. They were soaked in the primary antibody, a mouse monoclonal antibody against α-smooth muscle actin (Sigma; 1:400 concentration), for 2 hours at room temperature and then rinsed with PBS, pH 7.4. As a negative control, the slides were treated with mouse serum (Sigma; 1: 400 concentration) instead of the primary antibody. Any staining observed in the negative control tissue indicates the amount of nonspecific background staining. The biotinylated secondary antibody, goat anti-mouse immunoglobulin (Sigma; 1:300 concentration), was applied for 1 hour at room temperature. Following a rinse in PBS, the peroxidase reagent, or avidin-biotin complex (Sigma), was applied for 20 minutes at room temperature. The chromogen solution (Sigma) was applied for 15 minutes to develop the color of the stain. The slides were then rinsed, mounted, and coverslipped with glycerol gelatin (Sigma). Three regions of the sciatic nerve sections were analyzed: within the tissue capsule, within the nerve trunk, and within and around the tube walls.

Statistical methods

Two-factor analyses of variance (ANOVA) were performed to determine the effect of tube type and presence of the collagen-GAG matrix inside the tube on each outcome variable. If ANOVA indicated statistical significance, subsequent multiple comparisons were made using the Student Newman-Keuls test to determine differences between pairs of experimental groups (Zar, 1984). Following rejection of the null hypothesis of a one-factor ANOVA, Dunnett's test was used to compare each tube group to the normal and untreated controls (Zar, 1984). Student's t-test was used to determine if the changes from 30 to 60 weeks, in each implant group, were significant. To correct for possible errors using multiple t-tests, the Bonferroni approximation for multiple paired comparisons was used to adjust the critical P value by dividing the experimental P value ($P = 0.05$) by the number of t-tests performed (Lieber, 1994). Statistical significance was accepted for $P < 0.05$, and P values have been reported when the level of significance was higher.

TABLE 1. Frequency of Occurrence of the Three Tissue Types Observed within the Gap[1]

	No bridging tissue	Tissue cable (connective tissue only, no axons)	Nerve trunk (connective tissue with axons)	Success rate of regeneration (%)
No tube	5/5	0/5	0/5	0
SI	5/9	2/9	2/9	22
SI/M	0/8	0/8	8/8	100
LC	0/8	0/8	8/8	100
LC/M	0/9	0/9	9/9	100

[1]Data are presented as the number of animals in each category over the total number of animals evaluated. Untubulated animals had a 6-week survival time, whereas the data for the tubulated groups are from 30 and 60 weeks postoperatively. Regeneration was considered successful only when a nerve trunk bridged the gap. The success rate of regeneration is shown for each group.

RESULTS

Tissue types formed in the gap

When tissue bridged the 10-mm gap, it comprised either a connective tissue cable containing no axons or a nerve trunk. Nerve trunks comprised myelinated axons wrapped inside a connective tissue envelope. Verification of the presence of myelinated axons in nerve trunks that formed in all tubulated groups of this study was reported in detail previously (Chamberlain et al., 1998a,b). Regeneration was considered successful only when a nerve trunk formed within the gap.

The absence of a tube implant led to formation of a neuroma at the end of the injured proximal nerve stumps, and no bridging of the gap with connective or nervous tissue was observed in this group (Table 1). Implantation of a tube did not completely eliminate neuroma formation. In five of the nine animals implanted with an unfilled silicone tube (the SI group), a neuroma formed on the proximal nerve stump with similar gross characteristics to the untubulated group and no bridging tissue was observed (Table 1); in two of the animals, a thin connective tissue cable bridged the gap (Table 1); and in the remaining two animals, a nerve trunk formed between the nerve stumps, yielding a 22% regeneration rate (Table 1). In contrast, a nerve trunk connected the proximal and distal stumps in all of the matrix-filled silicone (SI/M; n = 8), unfilled collagen (LC; n = 8), and matrix-filled collagen (LC/M; n = 9) tube groups retrieved at 30 and 60 weeks (Table 1).

Nerve trunk diameter

The nerve trunk diameter in the middle of the nerve gap was dependent on tube composition (Fig. 2). In the empty and matrix-filled collagen tubes, the nerve trunk diameter in the middle of the gap increased significantly between 6 to 30 weeks ($P < 0.01$) and then decreased significantly between 30 and 60 weeks ($P < 0.01$). In contrast, the nerve trunk diameter in both silicone tube groups did not change significantly over the entire period of the study, i.e., 6–60 weeks ($P > 0.4$).

At 30 weeks, there was a significant effect of tube composition ($P < 0.001$) and no effect of matrix presence ($P > 0.3$) on the nerve trunk diameter in the middle of the nerve gap (Fig. 3). The LC and LC/M nerve trunks were signif-

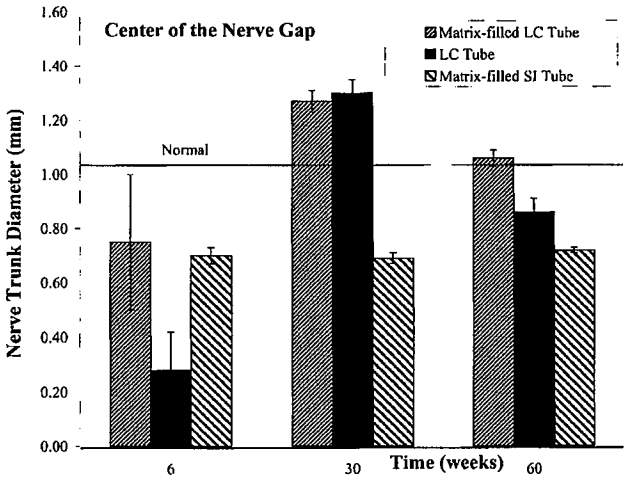

Fig. 2. Nerve trunk diameter in the center of the nerve gap for the matrix-filled silicone (SI/M), matrix-filled collagen (LC/M), and unfilled collagen (LC) tube groups plotted as a function of time. In the SI/M group, the nerve trunk diameter did not change significantly through the length of the study (6–60 weeks). In contrast, in the LC and LC/M groups it increased significantly from 6 to 30 weeks and then decreased significantly between 30 and 60 weeks.

icantly larger in diameter than the SI and SI/M nerve trunks (Fig. 3; $P < 0.001$). The SI/M and SI groups had similar nerve trunk diameters ($P > 0.3$); however, a nerve trunk formed in only two of nine SI animals compared with all eight animals in the SI/M group. Tissue cables, containing no axons, that formed inside the SI devices (in two of nine animals) had diameters significantly smaller than all of the nerve trunk diameters ($P < 0.01$). In the untubulated animals, no nerve trunks were formed.

In all experimental groups, including the neuroma group, the total tissue diameter in the proximal sciatic nerve stump was significantly higher ($P < 0.05$) than that of the normal nerve at 6 weeks (Fig. 4, Table 2). At 30 and 60 weeks, the tissue diameter in the proximal stump remained significantly higher than normal for all tubulated groups (Table 2). There were no significant differences among implant groups in the tissue diameter at the proximal sciatic nerve stump at 6, 30, and 60 weeks (Fig. 4a, Table 2; $P > 0.4$).

In the distal sciatic nerve stump, the group in which the neuroma had formed had a significantly larger tissue diameter than the normal nerve at 6 weeks (Fig. 4b, Table 2; $P < 0.05$). Similarly, at 30 and 60 weeks, the SI/M, LC, and LC/M groups had a significantly larger tissue diameter in the distal nerve stump than in the normal nerve ($P < 0.01$). At 30 and 60 weeks, the tissue diameter was significantly smaller for the SI group than for all other implant groups ($P < 0.001$) as well as for the normal nerve (Table 2; $P < 0.01$).

Tissue capsule thickness

The connective tissue capsule in the normal nerve, the perineurium, is a thin collagenous layer containing one to two cell layers that surrounds each fascicle (Fig. 3a). In the tubulated groups, a connective tissue capsule surrounded all nerve trunks at 30 weeks. All trunks were unifascicular in structure. Capsules in the collagen tube groups were thin collagen layers containing approximately one to two cell layers (Fig. 3b), similar to the normal perineurium. In contrast, the silicone-tubulated groups had a much thicker tissue capsule that contained approximately 15–20 cell layers and was continuous around the entire perimeter of the nerve trunk (Fig. 3c).

In the animals that received no tubulation, a thick tissue capsule, similar to that observed in the silicone tube groups, formed around both the proximal neuroma and distal tissue. However, instead of ensheathing the entire nerve trunk, as in the silicone tube groups, the connective tissue capsule capped off the neuromas (Fig. 4) and tissue at the distal stump. The outer layer of the tissue cap was covered with elongated cells that were fibroblast-like in appearance.

The thickness of the tissue capsule around newly formed tissue in the middle of the gap did not change significantly for any group during the period of investigation, i.e., 6–60 weeks ($P > 0.3$). At 30 weeks, tube composition had a significant effect on tissue capsule thickness (Fig. 5; $P < 0.001$), whereas the presence of the collagen-GAG matrix had no effect ($P > 0.5$). Both the SI and SI/M groups had significantly thicker tissue capsules than the LC and LC/M groups ($P < 0.001$). The tissue capsule thicknesses of the LC and LC/M groups were not significantly different from normal ($P > 0.4$). It was also observed that the thickness of the tissue capsule did not change significantly along the length of the nerve for any of the experimental groups ($P > 0.3$).

αSmooth muscle actin positive cells aligned circumferentially inside the tissue capsule

Smooth muscle cells lining blood vessels stained positive, serving as a positive control for the α-smooth muscle actin staining in each paraffin section. In the normal nerve, perineurial cells stained positively for α-smooth muscle actin (Fig. 6). Similarly, in nerves regenerated through collagen tubes (both unfilled and matrix-filled), a single layer of cells containing α-smooth muscle actin was observed at 30 and 60 weeks in some locations within the tissue capsules (Fig. 7a). However, unlike the normal nerve, the cell layer was not continuous around the entire length of the nerve trunk. Previous publications from our laboratory noted that no myofibroblasts were found in the collagen tube as early as 6 weeks (Chamberlain, 1996; Chamberlain et al., 1998a).

Cells that stained positively for α-smooth muscle actin were observed at both 30 and 60 weeks in the tissue capsules formed inside the silicone tubes (both unfilled and matrix filled; Fig. 7b); however, their appearance was much different than in normal perineurium. The thick tissue capsule was filled with at least 15–20 contractile cell layers that surrounded the entire perimeter of the nerve trunk. Similar findings had been reported 6 weeks postoperatively: all tissue capsules inside silicone tubes contained contractile cells (Chamberlain, 1996; Chamberlain et al., 1998a).

In the untubulated group, contractile cells were observed inside the tissue capsules that capped off both the proximal neuroma and the tissue at the distal stump (Fig. 8). In the neuroma, the cells located within the tissue capsule stained positive for α-smooth muscle actin along the entire length of the neuroma (parallel to the nerve axis); however, close to the capped end, almost all of the cells within the tissue cap stained positive (Fig. 8a). It appeared as if the tissue capsule converged (or contracted)

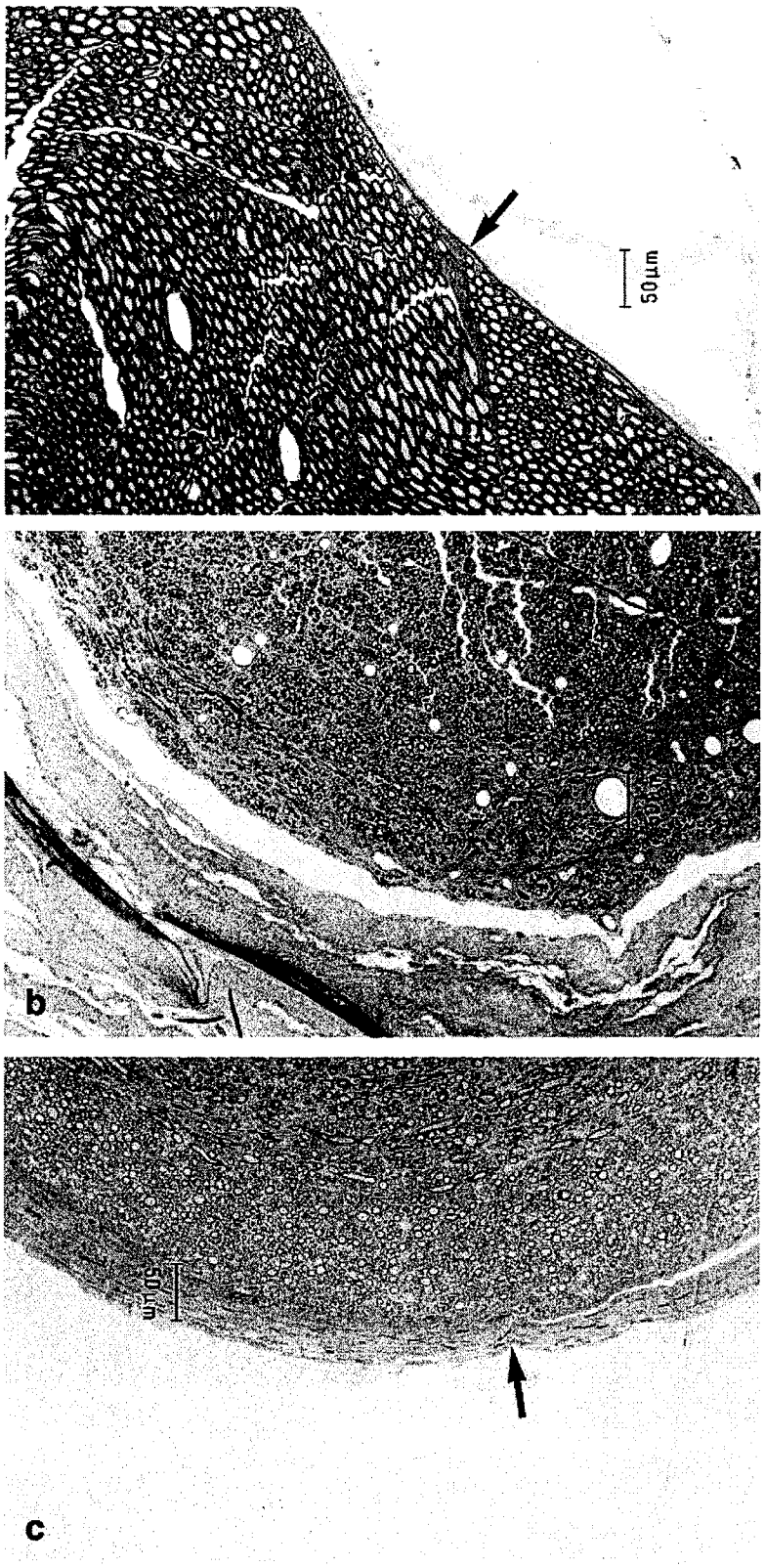

Fig. 3. Cross-sections of nerve tissue at 30 weeks postoperatively, postfixed with osmium tetroxide and stained with toluidine blue. **a:** Normal nerve tissue. The tissue capsule, perineurium (arrow), was a very thin collagenous layer containing only one to two cell layers. **b:** Tissue from the center of the nerve gap (5 mm from the proximal cut) of a matrix-filled collagen tube (LC/M). Similar to the normal nerve, the tissue capsule was thin (approximately 4 µm) and contained only one to two cell layers. The undegraded collagen tube is visible (LC). **c:** Tissue from the center of the nerve gap of a matrix-filled silicone tube (SI/M). The tissue capsule was thick (approximately 50 µm) and contained at least 15–20 cell layers at every location. In the silicone tubes, the tissue capsule buckled (arrow) at localized sites. Scale bars = 50 µm.

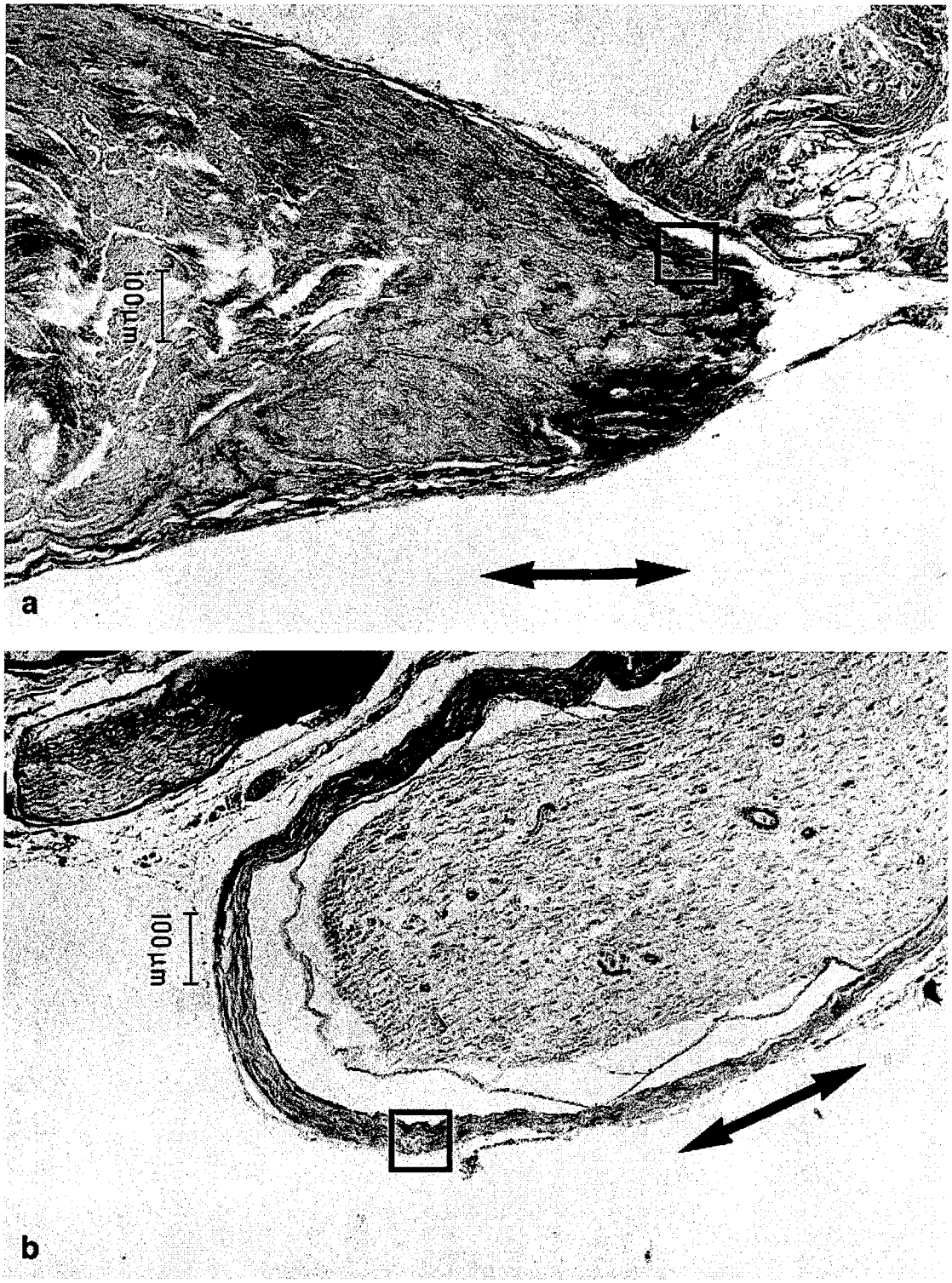

Fig. 4. Longitudinal sections of the proximal neuroma and tissue at the stump formed within untubulated gaps and retrieved after 6 weeks. The tissue is stained with Masson's trichrome. The arrow indicates the direction of the nerve axis. **a:** In the proximal neuroma, a thick, collagen tissue capsule (stained blue) surrounded the nerve tissue and then converged to form a cap at the end of the neuroma. The dense collagenous tissue formed at the end of the neuroma resembled fibrous scar tissue. The tissue capsule around the nerve stump was approximately 20–50 µm thick. The black box outlines the area of tissue shown under high magnification in Figure 8a. **b:** In the distal stump, a similar collagen tissue capsule was visible, approximately 50 µm thick, which capped off the distal nerve stump. The black box outlines the area of tissue shown under high magnification in Figure 8b. Scale bars = 100 µm

TABLE 2. Tissue Diameter (mm) in the Proximal and Distal Nerve Stumps[1]

	Proximal sciatic nerve stump			Distal sciatic nerve stump		
	6 weeks	30 weeks	60 weeks	6 weeks	30 weeks	60 weeks
Normal		0.85 ± 0.02			0.83 ± 0.02	
Neuroma	1.4 ± 0.2	—	—	1.5 ± 0.1	—	—
SI	1.4 ± 0.1	1.1 ± 0.3	1.6 ± 0.2	—	0.5 ± 0.1	0.5 ± 0.1
SI/M	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	—	1.0 ± 0.1	1.1 ± 0.1
LC	1.3 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	—	1.2 ± 0.1	1.2 ± 0.1
LC/M	1.5 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	—	1.1 ± 0.1	1.0 ± 0.1

[1]Diameters of the proximal sciatic nerve stump (−1 mm from the proximal cut) and the distal sciatic nerve stump (11 mm from the proximal cut) at 6, 30, and 60 weeks. The neuroma (untubulated group) was only evaluated at 6 weeks postoperatively; therefore, no data are presented for 30 and 60 weeks. In addition, the diameters of the distal sciatic nerve stump were not available for the tube groups at 6 weeks. In the proximal nerve, all treatment groups had a significantly larger tissue diameter than the normal nerve at 6, 30, and 60 weeks. In the distal stump, there was a significantly larger tissue diameter than normal. In contrast, by 60 weeks in the SI group, which had no regeneration of axons across the gap, the diameter was significantly smaller than normal.

until it ultimately formed a cap over the injured tissue. In the distal scar, the tissue cap was similar; however, the capping tissue surrounded the distal cut end with apparently less axial outgrowth of tissue. In this case, the capping connective tissue was not intermingled with the nerve trunk; rather, it appeared to form a distinct layer (Fig. 8b).

Cells aligned axially within the nerve trunk

In all experimental groups, cells containing α-smooth muscle actin were observed within the bulk of the nerve trunk at 6, 30, and 60 weeks, aligned parallel to the nerve axis. In some cases, the myofibroblasts constituted as many as 20% of all nonneuronal cells. The contractile cells were observed to be aligned parallel to the nerve axis, in both cross-sections and longitudinal sections (Fig. 9). There were no apparent differences in the presence or quantity of axially aligned contractile cells among groups, indicating that neither the presence of the collagen-GAG matrix nor the tube composition had an effect. It has been previously reported that, at 6 weeks, the myofibroblasts were most notable in the distal portion of the nerve gap, a

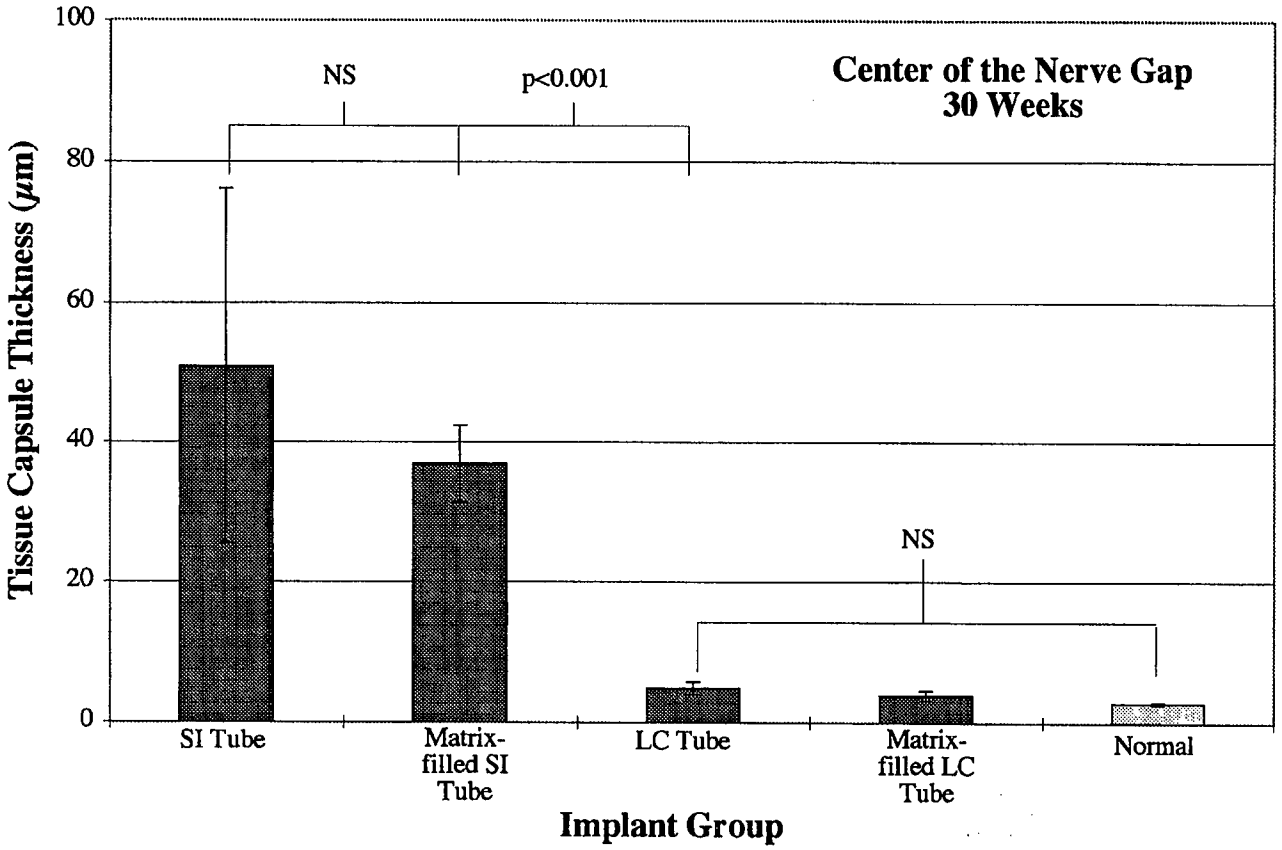

Fig. 5. Tissue capsule thickness in the center of the nerve gap for all implant groups at 30 weeks. The tissue capsules in the silicone tubes were significantly thicker than those in the collagen tubes ($P < 0.001$). The tissue capsule thicknesses in the collagen tubes were not significantly different from normal nerve. NS, not significant.

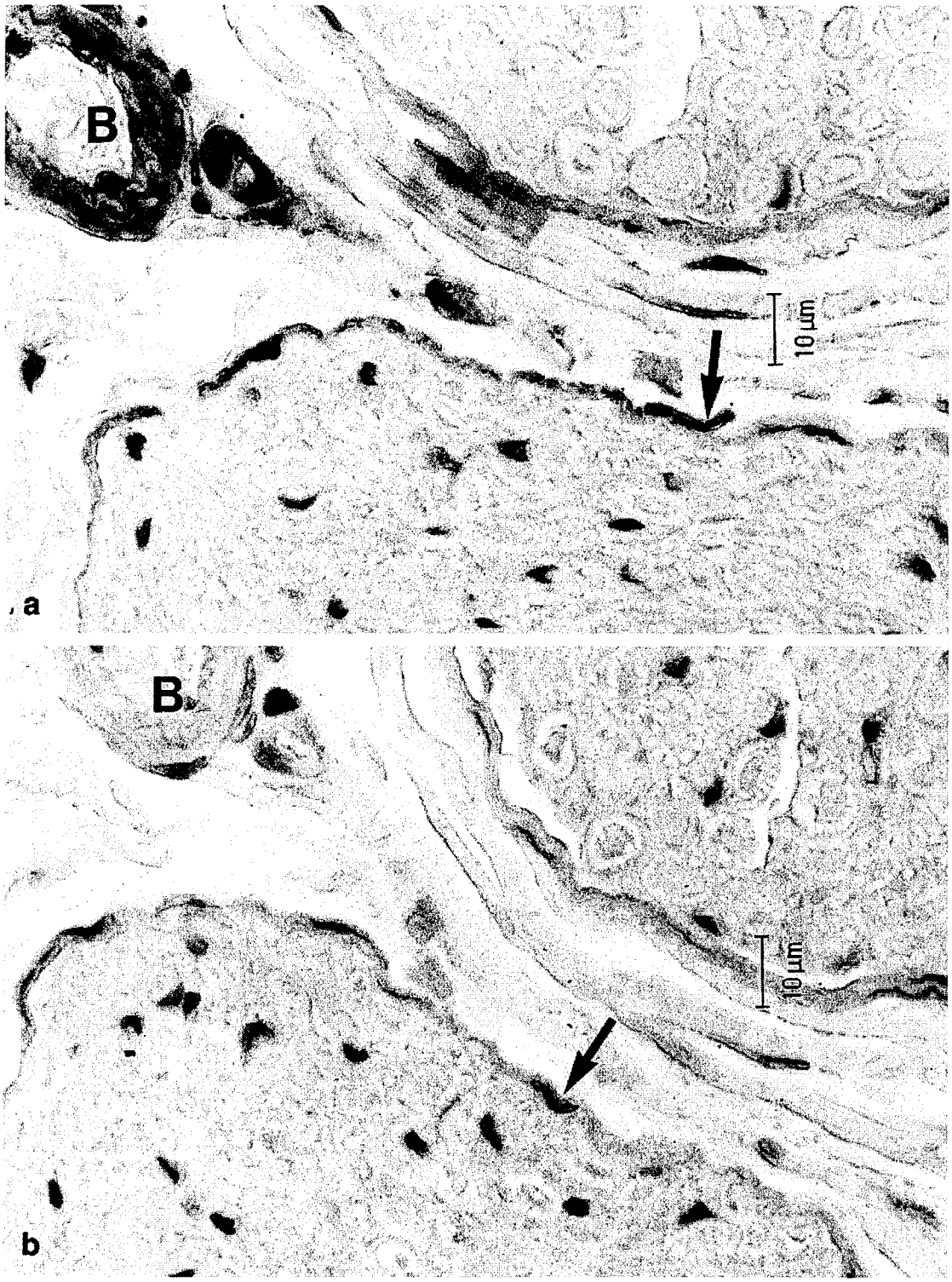

Fig. 6. Cross-sectional serial micrographs of normal nerve tissue retrieved after 30 weeks and stained with (a) the α-smooth muscle actin antibody and (b) the corresponding negative control treated with mouse serum. Positive staining for α-smooth muscle actin is red/brown in color. **a:** The cells of the normal perineurium (arrow) stained positively for α-smooth muscle actin, indicating that they may have contractile properties. The blood vessel (B) is a positive control for the stain. **b:** The negative control of the same tissue section shows no specific staining of the perineurial cells (arrow) or the blood vessel (B) and provides comparison for the amount of nonspecific background staining to be expected with the immunohistochemical method. Scale bars = 10 µm

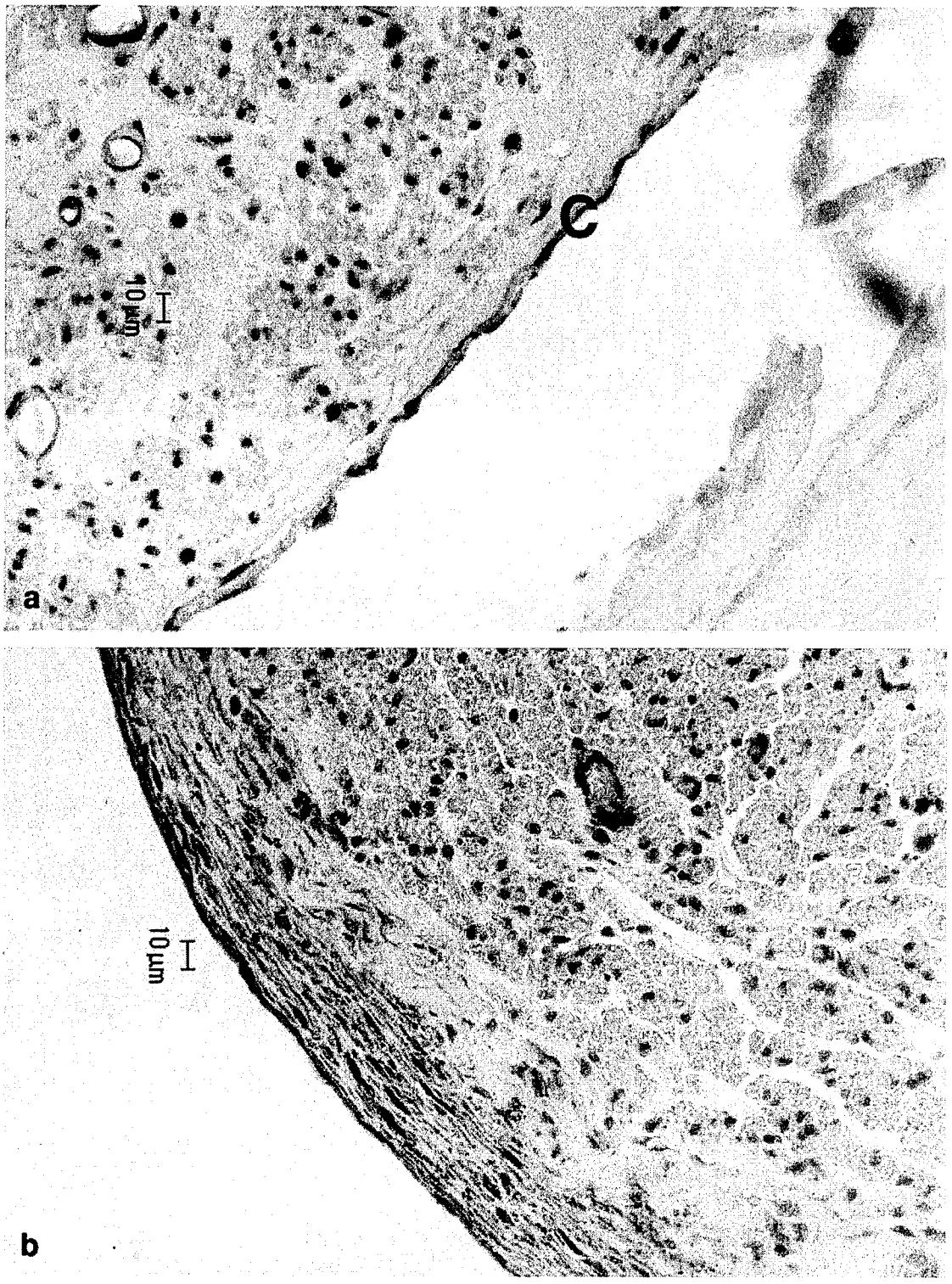

Fig. 7. Cross-sectional micrographs of nerve tissue retrieved after 60 weeks. Both micrographs were stained with the α-smooth muscle actin antibody and viewed at the same magnification. **a:** Matrix-filled collagen tube device. The cells of the tissue capsule are stained positively; however the contractile cells (C) are not confluent around the entire nerve trunk. In all areas, the contractile cells were no more than one to two cell layers thick. **b:** Matrix-filled silicone tube device. In contrast to the collagen tube, the SI/M devices had approximately 15–20 cell layers of contractile cells around the entire perimeter of the nerve trunk. Scale bars = 10 μm.

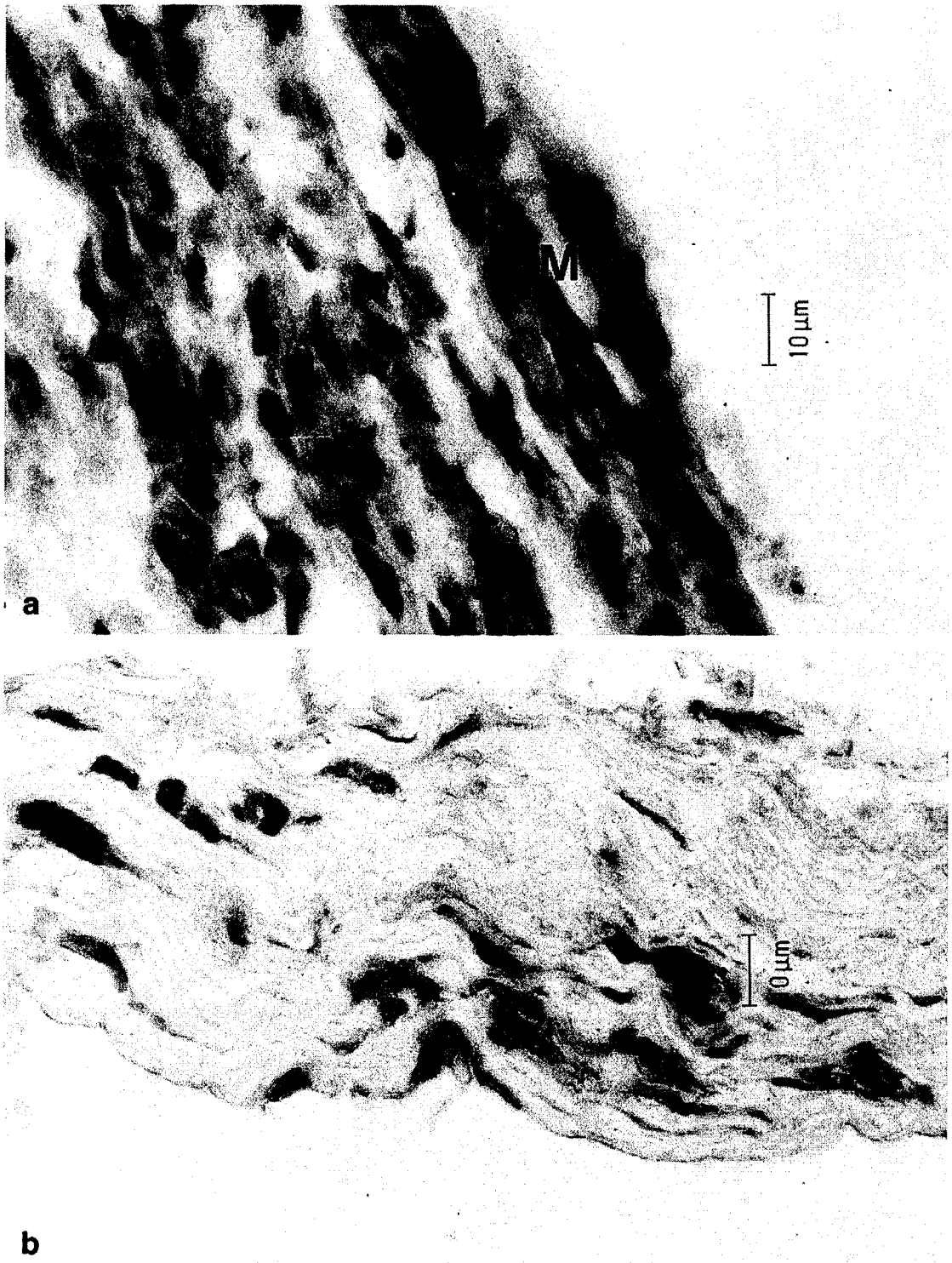

Fig. 8. Longitudinal sections from untreated animals retrieved at 6 weeks and stained with the α-smooth muscle actin antibody. **a:** Tissue from the tip of the proximal neuroma (see Fig. 4a for exact location). Almost every cell stained positively for α-smooth muscle actin in this region (both brown and red indicate positive staining). The cells along the edge of the tissue cap are myofibroblasts (M). An epithelial layer surrounding the tissue cap is not apparent at 6 weeks. **b:** Tissue from the tissue cap at the distal site (see Fig. 4b for exact location). Myofibroblasts were abundant and the connective tissue appears to be buckling, which would suggest the cells are contracting. Buckling was consistently present in the silicone-tubulated and no-tube groups, but absent from the collagen tube groups, suggesting the buckling was not a histological artifact. Scale bars = 10 µm.

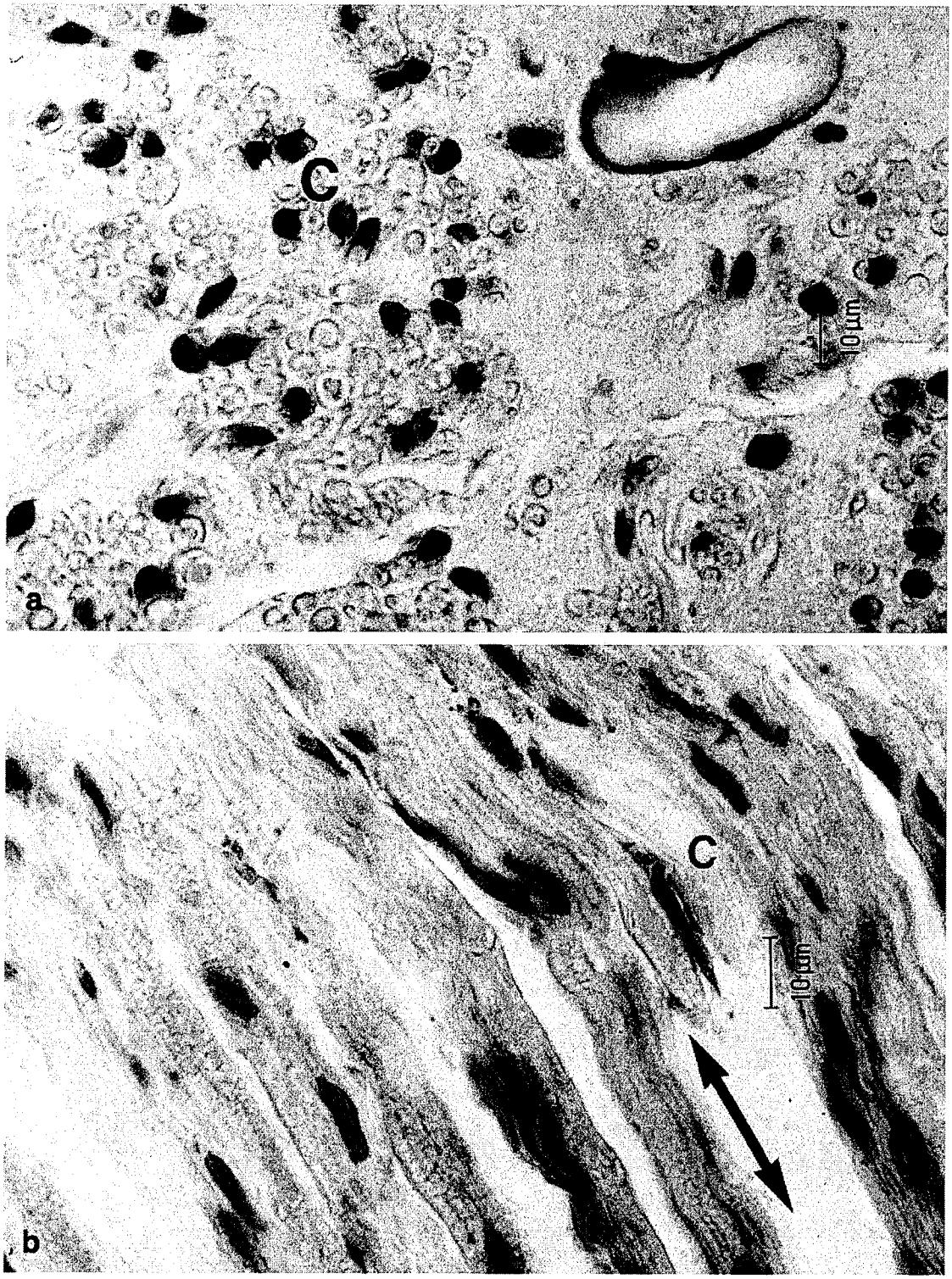

Fig. 9. Nerves regenerated through a matrix-filled collagen tube implant at 30 weeks postoperatively and stained with the α-smooth muscle actin antibody. **a:** Cross-sectional micrograph showing contractile cells (C) that have been cut transversely. The contractile cells are aligned in the same direction as the axons, coming out of the page in this case. **b:** Longitudinal micrograph showing the axially aligned contractile cells (C) in longitudinal view. The arrow indicates the direction of the nerve axis. Both micrographs are at the same magnification. Scale bars = 10 μm.

location coincident with the advancing axonal front (Chamberlain, 1996).

Cells aligned circumferentially inside the tube wall or outside the tube

Contractile cells were observed inside the wall thickness of the collagen tubes at 30 weeks (Fig. 10a). The long axes of the contractile cells were aligned in a circumferential direction around the tube wall. The cells did not form a continuous layer within the tube wall; however, in several locations, multiple cells had formed apparent connections (Fig. 10a). At 6 weeks, no contractile cells were previously reported within the walls of collagen tubes (Chamberlain, 1996; Chamberlain et al., 1998a). Immediately outside the silicone tubes, a tissue layer (approximately 40 μm thick) was present at 30 and 60 weeks. In all cases, it contained approximately 15–20 cell layers composed of circumferentially aligned contractile cells (Fig. 10b). A similar capsule was previously observed on the outside surface of the silicone tubes as early as 6 weeks postoperatively (Chamberlain, 1996; Chamberlain et al., 1998a).

DISCUSSION

Identification of myofibroblasts

The observation of contractile cells in the normal nerve perineurium in this study is consistent with an early report of filaments in perineurial cells in the mouse sciatic nerve that were similar to those observed in the smooth muscle (Ross and Reith, 1969). It is also consistent with ultrastructural studies in which bundles of closely aggregated filaments, similar in appearance to the myofilaments of smooth muscle, were observed in the cytoplasm of perineurial cells (Thomas and Olsson, 1975). Fibroblasts containing bundles of 5–7-nm filaments and having an appearance reminiscent of myofibroblasts found in granulation tissue were later reported by electron microscopy in the transected mouse sciatic nerve (Scaravilli, 1984). Identification of α-smooth muscle actin in the filaments has, however, not been reported. Masur et al. (1996) have suggested that the observation of α-smooth muscle actin, identified by immunohistochemical methods, is definitive proof that cells are capable of exerting contractile forces. Unlike ultrastructural observations that identify filamentous actin structures, positive staining with the α-smooth muscle actin immunohistochemical stain verifies that the actin within the cells is the α-smooth muscle isoform, capable of inducing contraction. The staining also identifies the presence of the α-smooth muscle actin monomer, which is the precursor of filament formation (Masur et al., 1996). Recent studies have led to characterization of the sequence corresponding to the epitope of the monoclonal antibody against α-smooth muscle actin and have shown that this sequence is important for α-smooth muscle actin polymerization (Chaponnier et al., 1995).

Effect of tubulation

A comparison of the untreated group (neuroma) and the tubulated groups showed that tubulation typically resulted in successful formation of a nerve trunk connecting the proximal and distal stumps (Table 1). When the nerve stumps were not tubulated, a fibrous scar cap was formed over the ends of the nerve stumps, likely preventing formation of a tissue cable bridging tha gap. However, implantation of a tube did not always lead to nerve trunk formation between the nerve stumps, as evidenced by the very low regeneration rate in the unfilled silicone devices (Table 1). This is consistent with the conclusion that the limit of regeneration in this silicone-tubulated animal model is 10 mm (Lundborg et al., 1982a).

Effect of tube type

Whenever a tissue cable or nerve trunk bridged the nerve gap, a contractile capsule, similar to the one responsible for capping off the neuroma, was observed surrounding the nerve trunk along its entire length along the gap. Tube type had a significant effect on the nerve trunk diameter in the middle of the nerve gap (Fig. 2); nerve trunks that had regenerated inside silicone tubes had significantly smaller diameters than those formed inside collagen tubes.

The combination of necking in the nerve trunk at the center of the gap bridged by the silicone tube and the presence of a thick tissue capsule containing multiple layers of contractile cells suggests that the capsule restricts nerve growth and may apply contractile forces to the nerve trunk along its entire length. A similar contractile capsule has been reported to exert significant contractile forces around silicone breast implants (Rudolph et al., 1978). Additional evidence that supports this constriction model includes the uniformly circular shape of the nerve trunk cross-sections and the observation of buckling in the tissue capsules of nerves regenerated through the silicone tubes (Fig. 6). It is hypothesized, therefore, that the restrictive forces caused by the contractile capsule prevent the growth of axons in diameter, leading to the significant decrease in axon maturation observed previously in silicone tubes, compared to collagen tubes (Chamberlain et al., 1998b).

The differences in connective tissue response between collagen and silicone tubes could have been due to their known differences in chemical composition, permeability, or degradability. In the time period evaluated in this study, the collagen tubes remained undegraded; therefore, degradability was not a variable. It can be hypothesized that a collagen tube may inhibit formation of a contractile capsule by binding myofibroblasts on the interior surface of the tube through specific cell-matrix interactions (fibronexi; see review in Hynes, 1990); such binding would not be available to silicone tubes. According to this hypothesis, bound myofibroblasts lose their large-scale organization, which normally leads to formation of coherent, macroscopic bands that exert circumferential stresses around the nerve stumps and constrict the regenerating nerve fiber. This hypothetical process is similar to one used to explain a large body of data on the relation between inhibition of formation of a well-organized cluster of myofibroblasts and strong inhibition of skin wound contraction by formation of specific cell-matrix interactions (Yannas et al., 1989; Yannas, 1998).

Effect of collagen-GAG matrix

The collagen-GAG matrix was much more effective in the silicone group where its presence resulted in more frequent formation of a nerve trunk within the gap (Table 1). Filling the silicone tube with the collagen-GAG matrix increased the success rate of regeneration from 22% to

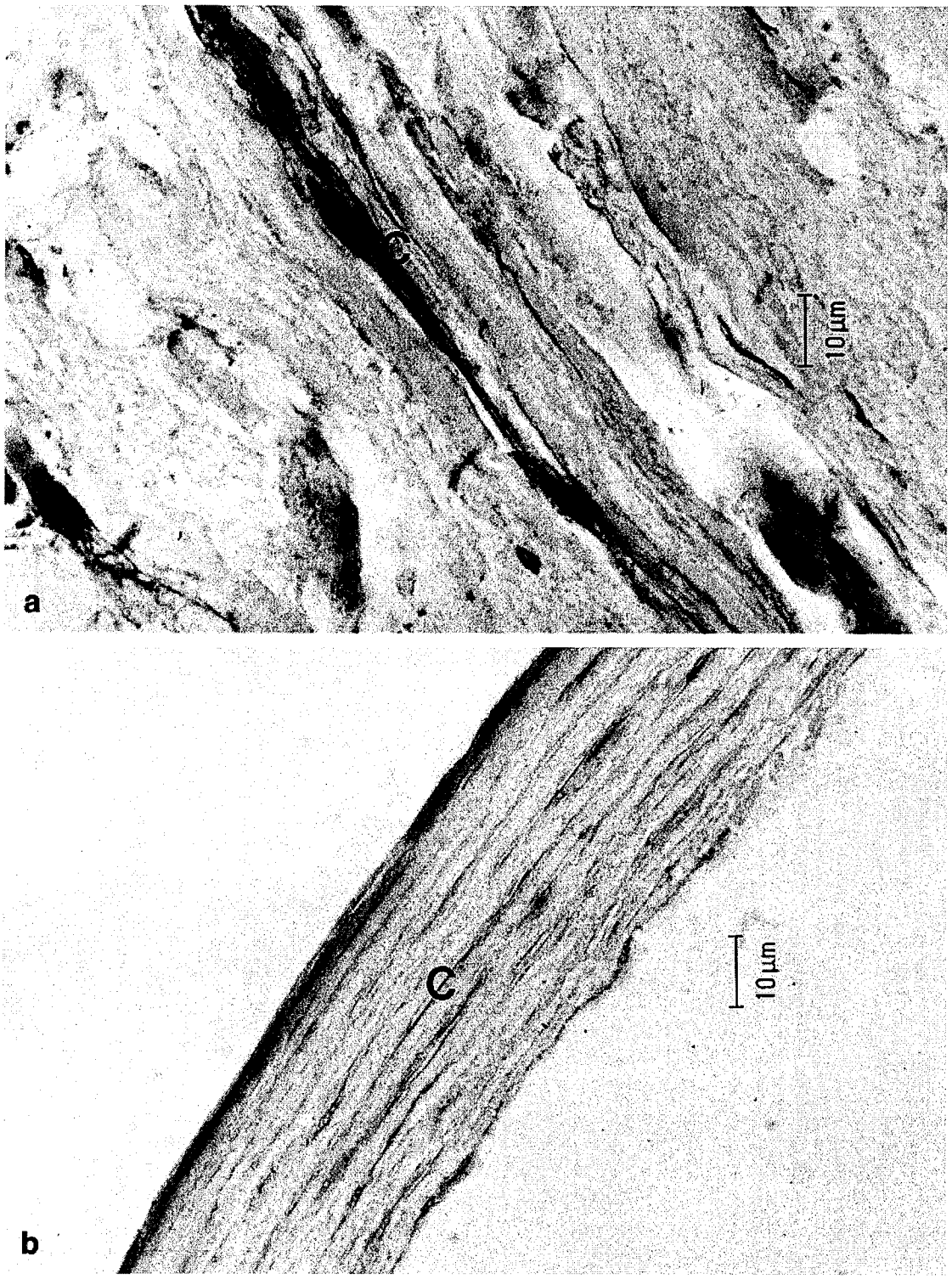

Fig. 10. Tissue sections retrieved after 30 weeks of implantation and stained with the α-smooth muscle actin antibody. **a:** Cross-sectional micrograph showing contractile cells (C) located between the lamellae of the collagen tubes. The contractile cells are aligned circumferentially. **b:** Cross-sectional micrograph showing the contractile cells (C) in the tissue layer formed around the outside of the silicone tube implants. Again, the contractile cells are aligned circumferentially. Both micrographs are at the same magnification. Scale bar = 10 μm.

100%. This finding confirms previous reports of improved success in regeneration across a 15-mm gap in the rat sciatic nerve following filling of silicone tubes with a collagen-GAG matrix similar, but not identical to, the one used in the present study (Yannas et al., 1987). Successful regeneration also occurred across a 10-mm gap when silicone tubes were filled with the collagen-GAG matrix used in the present study (Chang et al., 1990; Chang and Yannas, 1992; Chamberlain et al., 1998b). In the collagen tubes, the success rate was 100% both in the absence and the presence of the collagen-GAG matrix; in addition, the nerve trunk diameter in the middle of the gap inside the collagen tubes reached apparently maximum values independently of the presence or absence of the collagen-GAG matrix. The thickness of the tissue capsule and the number of contractile cell layers, however, were similar for the SI (unfilled silicone tube) and SI/M (silicone tube filled with the collagen-GAG matrix) groups. This suggests that the contractile forces generated by the tissue capsules may be similar for both groups.

These observations suggest that the presence of the collagen-GAG matrix does not reduce the contractile forces; rather, in order to explain the higher incidence of successful regeneration in the collagen-GAG matrix-filled silicone tubes (Table 1), it is hypothesized that the matrix presence may lead to early axonal elongation within the gap prior to confluence of the contractile capsule.This hypothesis is also consistent with the superior electrophysiological performance of regenerates in collagen-GAG-filled silicone tubes relative to unfilled controls (Chamberlain et al., 1998b).

New mechanism for nerve fiber regeneration across a tubulated gap

Based on the results of this study, a new model of nerve regeneration across a tubulated gap is proposed. It consists of the balance between two competitive forces: the axial forces created by the outgrowth of axons and nonneuronal cells from the proximal stump and the constrictive forces imposed by the tissue capsule that promote wound closure at the stump site. In the absence of an implant, a tissue capsule, containing many contractile cells, forms around the nerve trunk and ultimately caps off the cut nerve ends, closing the wound. Mechanisms of wound closure in other organs, primarily skin, in which myofibroblast layers play a critical role in transferring mechanical stresses over the scale of the wound have been reported (Rudolph et al., 1992; Yannas, 1998). However, a mechanism of wound closure has not been reported for the transected peripheral nerve.

Axially aligned contractile cells

The function of the axially aligned contractile cells is not understood; it has been hypothesized that these cells may be responsible for aligning newly synthesized collagen fibers and possibly regenerated axons during healing (Chamberlain, 1996). A similar process of collagen fiber alignment following transection injury has been described in the regenerating ligament tissue (Faryniarz et al., 1996). This hypothesis is strengthened by the observation of contractile cells at 6 weeks in register with the advancing axon front. There is significant evidence that, in vitro, axons elongate in response to axial tension applied to the axon growth cone (Bray, 1984, 1987; Dennerll et al., 1989; Zheng et al., 1991). Weiss (1944) has suggested that tubulation of transected nerve stumps trasmits tension between the stumps and the newly forming regenerate.

Another possible function of the axially contractile cells may be to facilitate closure of the wound by shortening the nerve gap. Tonge et al. (1996) observed that, after formation of a cellular bridge at 1 week, a nontubulated 2-mm gap in the mouse sciatic nerve had been reduced in length by 18%. This may implicate the contractile cells in shortening of the nerve gap. Additional information is necessary to determine if axially aligned contractile cells play a role in shortening the nerve gap or enhancing axon elongation across the gap. Further studies are required before the relation between wound closure mechanisms and peripheral nerve regeneration can be clarified in detail.

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Effects of Bridging a Gap in the Rat Spinal Cord with a Collagen Tube and Membrane

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Statement of Purpose: Recent work [1] has shown that an off-the-shelf collagen tube filled with a collagen sponge-like scaffold exceeds the performance of a nerve autograft, the "gold standard," in a rat model The objective of this study was to begin to apply this approach to the spinal cord using an absorbable type I collagen tube and to extend the concept by using a collagen membrane as a more easily applied wrap around the defect to protect the lesion from the collapse of surrounding tissue into the gap and to contain endogenous agents; specific attention was focused on the evaluation of the amount, organization and orientation of the fibrous scar in the defect.

Methods: A 5-mm mid-thoracic gap was created surgically in the rat spinal cord, as has previously been described [2,3]. There were 2 treatment schemes: 1) the ends of the cord were fitted into a collagen tube, and 2) a collagen membrane was wrapped around the cord stumps to contain the defect. A collagen membrane was also used along with both strategies as a dorsal barrier in order to further reduce scar infiltration of the defect. There were 5 experimental groups (n=4):

I. Non- implanted control group
II. Dorsal barrier only
III. Collagen tube
IV. Collagen tube and dorsal barrier
V. Collagen membrane wrap and dorsal barrier

Collagen tubes were fabricated by freeze-drying a type I microfibrillar collagen (from bovine tendon; Integra Life Sciences, Plainsboro, NJ) slurry that was injected into polytetrafluoroethylene (PTFE) molds into which were inserted PTFE coated glass rods, 3 mm in diam. The tubes were dehydrothermally treated at 120°C for 24 hr. The collagen membrane comprised porcine type I/III collagen (Geistlich Biomaterials, Wolhusen, Switzerland). Histomorphometric evaluation was performed 4 weeks post-implantation. Specimens were fixed in formalin. Sections of osmium-postfixed, Epon-embedded tissue were stained with toluidine blue; sections of paraffin embedded tissues were stained with Masson's trichrome for collagen. Immunohistochemistry was performed using antibodies to anti-glial fibrillary acidic protein (GFAP) to detect astrocyte proliferation and α-smooth muscle actin (SMA) to reveal myofibroblasts.

Results/Discussion: All animals lost hindlimb function distal to the insult but maintained adequate forelimb mobility, grooming and consumption of food and water (provided *ad libidum*). As described previously [2,3], temporary loss of the reflex bladder voiding function required manual expression of the bladders.
The excised spinal cord tissue of every animal group, including Groups I and II, revealed a cord of reparative tissue that bridged the gap between rostral and caudal spinal cord stumps. Grossly, all of the collagen implants showed some degree of resorption. The collagen tubes exhibited fragmentation; it is not clear at what time postoperatively there may have been a loss of entubulation of lesion. Histological evaluation revealed that buckling of the wrap resulted in a degree of collapse into the lumen of the gap.
GFAP and SMA expression in the groups receiving the dorsal barrier was notably less than Groups I or III. Group II expressed the highest levels of GFAP and Group V the least among the groups with a dorsal barrier. SMA expression was minimal and indistinguishable between Groups IV and V, but significantly higher in Group II. Pronounced fibrous and glial scar formation was found in the groups with no dorsal barrier: control group (I) and the group with the collagen tube, but no dorsal barrier (III); the least scar in the tube and wrap groups with the dorsal barrier (IV and V). The most axons were found in the tube and wrap groups with the dorsal barrier (IV and V; Figure 1), but the numbers were substantially less than normal (>100,000 axons). The majority of the axons were less than 5 μm in diameter.

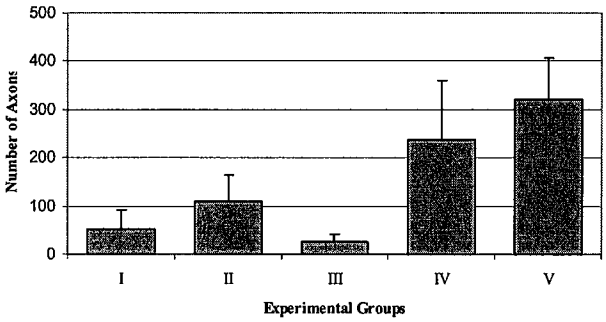

Figure 1. Number of axons in the center of the defect. Mean ± std. dev.

Conclusions: The combination of a collagen membrane wrapped around a spinal cord gap and a dorsal barrier may be effective in reducing the formation of fibrous scar in gaps in the spinal cord and thus may contribute to creating a hospitable environment for a regenerative process, which will likely require the implantation of antagonists of nerve growth inhibitors, neurotrophic factors, and stem cells.

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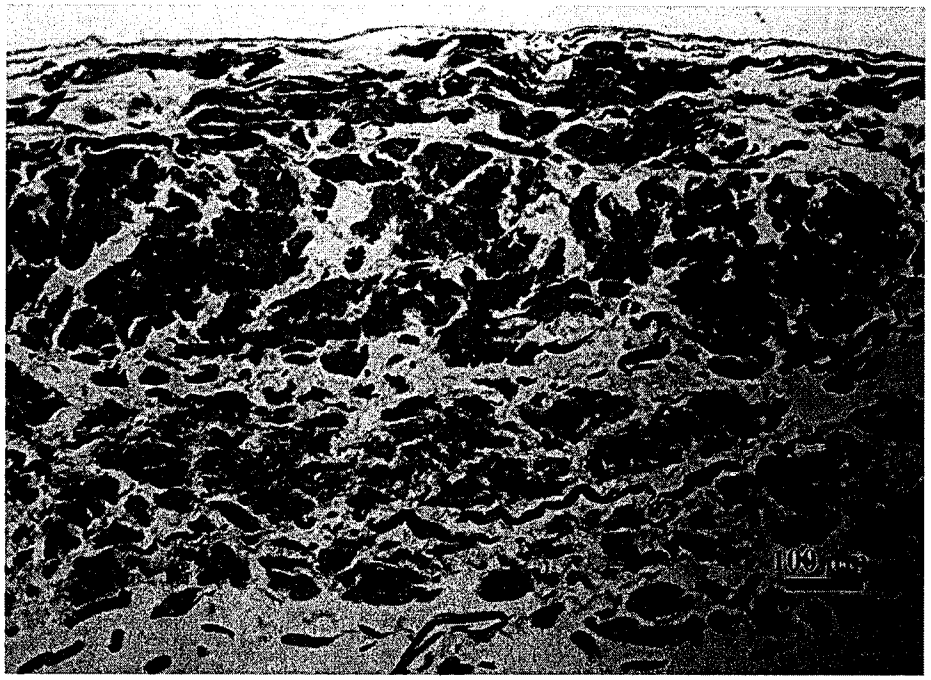

Fig. 1. Light micrograph showing a cross-section through the BioGide® material with the compact smooth barrier side at the top and the soft fibrous side at the bottom. Sample embedded in a plastic resin, JB-4. Section satined with analine blue.

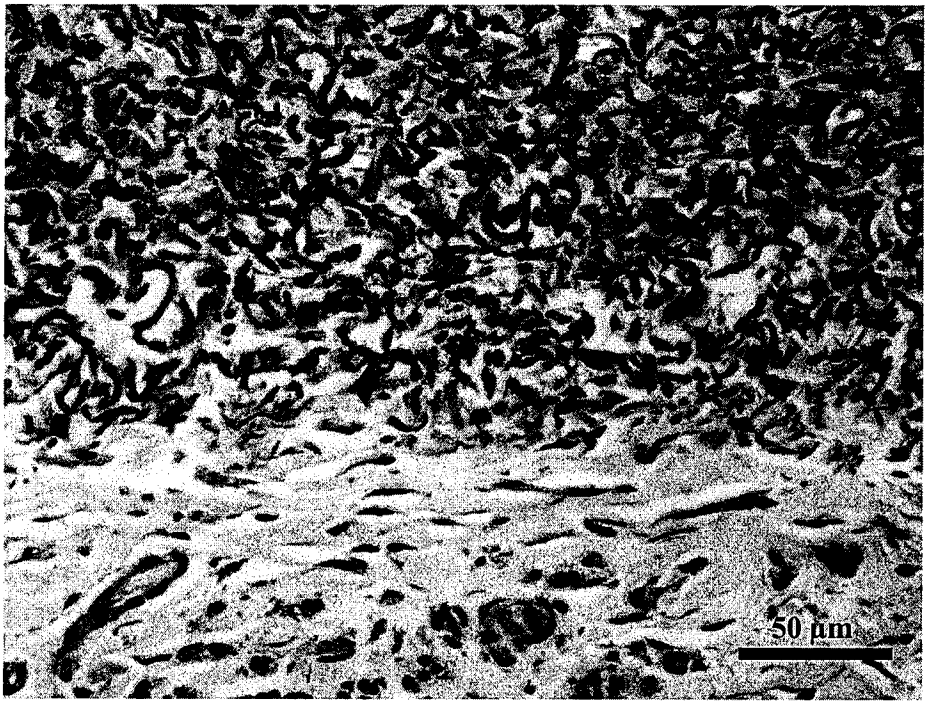

Fig. 2. Histology showing tissue and cells infiltrating the soft fibrous side of the BioGide® material entubulating a gap in a nerve (rat spinal cord), 4 weeks after implantation. Sample embedded in paraffin. Section satined with hemaoxylin and eosin.